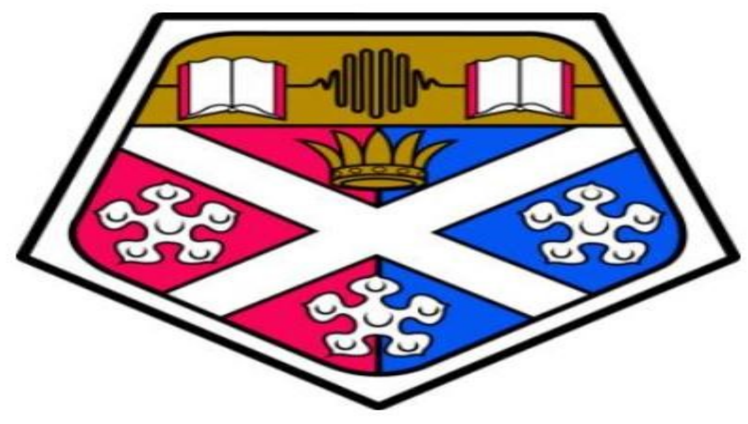

University of Strathclyde

Strathclyde Institute of Pharmacy and Biomedical Science

DEVELOPMENT OF RAPID SCREENING ASSAYS FOR THE DETECTION OF FOODBORNE PATHOGENS IN FOOD

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Thesis presented in fulfilment of the requirements for
the degree of Doctor of Philosophy

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Declaration

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Abstract

Fast and simple detection methods of bacteria and viruses in food samples have become a basic requirement in many fields, such as food production. Most rapid methods depend on immunological techniques and bio-sensing rather than the traditional culture based techniques. The purpose of this study was to develop numerous fast, simple and cheap assays to detect pathogenic bacteria and viruses in food samples and food processing plants, the methods are based on different scientific principles, such as immunological techniques with specific binding elements (antibodies and aptamers) or general capturing elements (lactoferrin), in addition to molecular methods, such as loop mediated isothermal amplification (LAMP), in the first approach, a simple and reliable colorimetric immune sensor using specific antibodies was developed and evaluated as a novel and rapid detection platform for foodborne pathogenic bacteria on surfaces of poultry processing plants, cotton swabs were activated by aldehyde groups to be used as a substrate and pre-concentration matrix for pathogens. The assay was tested on artificially contaminated surfaces with different concentrations 10^{1}-10^{8} CFU/ml of *Salmonella typhimurium, Salmonella entritidis, Staphylococcus aureus* and *Campylobacter jejuni.* In the second approach, the rapid detection of *S. Typhimurium, S. entritidis, S. aureus, C. jejuni* in addition to *Norovirus* was achieved using a colorimetric immune sensor, this method can be used for the on-site detection of pathogenic bacteria on the surfaces of chicken meat. In this assay, activated cotton swabs coupled with lactoferrin were used for pre-concentrating pathogenic bacteria from the contaminated chicken

surfaces, the color intensity of the cotton surfaces increased with the increasing concentration of the pathogenic bacteria. The detection limit was found to be as low as 10 CFU/ml for *S. entritidis*, 100 CFU/ml for *S. entirica serovar typhimurium* and *C. jejuni* and 1000 CFU/ml for *S. aureus.* This method is highly specific and was further confirmed by the LAMP method, the same technique was used to detect *Norovirus* in food samples. In the third approach, a combination of the LAMP technique and nanotechnology was used for the detection of *Salmonella*, *E. coli.* 0157H and *C. jejuni* in poultry processing plants. Lactoferrin was used as a cross linker between the amplified DNA sequences and with less than 50 nm containing carboxylic acid functional groups, stainless-steel surfaces were artificially contaminated with different concentrations of bacterial cultures in the range 10 to 10^8 CFU. Positive samples were visually detected by observing the aggregation of dyed nanospheres forming a disc near the top of solution. Conversely, negative samples were characterised by dispersed dye in the solution. This assay showed very good sensitivity, ranging between 10 CFU in both *Salmonella* and *E. coli,* and 100 CFU in the case of *C. jejuni*. The last approach was a fluorescence-based study for mapping the highest affinity truncated aptamers from the full-length sequence and its integration in a graphene oxide platform for the detection of *S. enteritis* to identify the best truncated sequence. Molecular beacons were used as well as a displacement assay design. The detection limit of the aptasensors fabricated using the truncated aptamer was lower than the full-length aptamer. Moreover, the aptasensors did not show significant cross reactivity with other related bacteria such as *S. typhimurium, S. aureus*

and E. coli. The fluorescent/graphene oxide aptasensors also demonstrated good recovery for the detection of *S. entirica serovar enteritidis* from spiked milk samples.

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3. List of abbreviations

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1	LAMP	Loop mediated isothermal amplification.
2	LF	Lactoferrin.
3	CFU	Colony forming unit.
4	FBD	Foodborne diseases.
5	GAP	Good agriculture practice.
6	GMP	Good manufacturing practices.
7	CDC	Centre for disease and control.
8	RT-PCR	Real time polymerase chain reaction.
9	qPCR	Quantitative polymerase chain reaction.
10	NASBA	Nucleic acid sequence based amplification.
11	FCM	Flow cytometry.
12	FDA	Food and drug administration.
13	ELIZA	Enzyme-linked immune sorbent assay.
14	IMMPs	Immunomagnetic nanoparticles.
15	LMI	Lateral flow immunoassay.
16	LFD	Lateral flow devices.
17	MI	Microfluidic immunoassay.
18	IMS	Immunomagnetic separation.

19	RIA	Radioimmunoassay.
20	IF	Immunofluorescence.
21	DIF	Direct immunofluorescence.
22	IIF	Indirect immunofluorescence.
23	BSA	Bovine albumin serum.
24	EDC	1-ethyl (3- dimethylaminopropyl) carbodimide.
25	NHS	N-hydroxysuccinimide.
26	DIC	Dialdehyde cellulose.
27	GO	Graphene oxide.
28	Kd	Dissociation constant.
29	FRET	Fluorescence resonance energy transfer
30	SWCNTs	Single walled carbon nanotubes.
31	HNB	Hydroxynapthol blue
32	LOD	Limit of detection
33	EM	Electron microscopy.
34	PFU	Plaque forming unit.
35	Ig	Immunoglobulin.
36	TSB	Tryptone soy broth

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Chapter 1

Introduction & Literature Review

1.1. Introduction

Concerns for food safety have stimulated increasing consumer awareness of potential pathogen contamination of food resulting in human diseases (**Ramesh** *et al.,* 2002). Poultry meat products are highly desirable, digestible and nutritious. Additionally, they are low in price in comparison to beef and lamb. Poultry meat is comprised of about 20–23% protein (**Smith**, 2001). The estimated poultry production in Riyadh markets in Saudi Arabia is 164,000 tons per year (**Haider** & **El-Eid**, 2005). However, poultry meat is considered one of the most common foods containing foodborne pathogens (**Forsythe** & **Hayes**, 2002). Bacterial diseases are a serious problem for a nation's food supply and have been attributed to cross contamination with human pathogenic organisms such as *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Campylobacter jejuni* (**Knowles** & **Roller**, 2001; **Chia** *et al.,* 2009). The primary sources of bacterial contamination of poultry are faecal material and dirt on surfaces (**Dickson** & **Anderson**, 1992). The incidence of food poisoning in Saudi Arabia due to food of animal origin was 76.8 % in comparison to 23.2% due to food of plant origin. Poultry ranked first as a cause of food poisoning, with an incidence of 29.3%, followed by meat and cream, with an incidence of 15.3% and 8.8 % respectively. In the eastern region of the kingdom, such incidence reaches 96.3% for food of animal origin and 37.8% for poultry and their products (**Haider** & **El-Eid**, 2005).

1.2. Foodborne diseases (FBD)

Foodborne illnesses have been defined by the World Health Organization (WHO) as infectious and toxic diseases caused by agents that enter the body through the ingestion of food (**Velusamy** *et al.,* 2010). Indeed, many high risk human pathogens are transmitted through contaminated food and water (**Khan** *et al.,* 2014). There are 250 types of pathogens that cause foodborne illnesses, including bacteria, viruses, parasites, and prions (**Schmidt** *et al.,* 2009). However, the three most prominent disease-causing foodborne contaminants are *Campylobacter spp, Salmonella spp and L. monocytogenes* (**Chemburu** *et al.,* 2005; **Velusamy** *et al.,* 2010). Food cross contamination, poor sanitation, as well as the improper preparation of food in farms, slaughterhouses, food processing and food supermarkets are the major causes of foodborne illnesses; consequently, the reduction of foodborne disease lies upon the good practices for management of these entities (**Redmond** & **Griffith**, 2003).

There are methodically significant programs for the reduction of foodborne pathogens in the food industry, such as good agricultural practices (GAP), good manufacturing practices (GMP), hazard analysis and critical control point (HACCP) and food code indicating approaches (**Kay** *et al.,* 2008; **Mucchetti** *et al.,* 2008; **Jin** *et al.,* 2008; **Velusamy** *et al.,* 2010). Several outbreaks of bacterial pathogens such as *Salmonella Spp. C. jejuni, E. coli., Shigella spp., Y. enterocolitica, B. spp., Clostridium spp., S. aureus and L. monocytogenes* etc. can be linked to the consumption of various food sources worldwide every year (**Niessen** *et al.,* 2013). According to the Centre for Disease Control and Prevention (CDC), it has

been estimated that 48 million cases of foodborne illnesses occur in the United States (US) annually and approximately 128,000 cases require hospitalization and 3,000 cases result in death (**Scallan** *et al.*, 2011). Viruses are major causative agents for foodborne illnesses (59%), followed by bacteria (39%) and parasites (2%); however, bacterial agents are associated with the more severe cases, being responsible for most hospitalizations (63.9%) and deaths (63.7%) (**CDC**, 2010).

1.2.1. *Salmonella* spp

The avian micro-flora includes pathogens such as *Salmonella spp., C. jejuni, L. monocytogenes and E. coli.* (**Cochran** *et al.,* 2000; **Smith** *et al.,* 2005). *Salmonella* was named after Daniel Elmer Salmon, who first isolated *Salmonella Chloeraesuis* from pigs with swine cholera in 1884 (**Humphrey**, 2000). *Salmonella* spp. are members of the family *Enterobacteriaceae*, comprising of two species, *Salmonella enterica and Salmonella bongori*. *S. enterica* consists of six subspecies: (1) *S. enterica* subspecies *enterica*, (2) *S. enterica subsp. salgamae, (3) S. enterica subsp. arizonae, (4) S. enterica subsp. diarizonae, (5) S. enterica subsp. houtenae,* and *(6) S. enterica subsp*. indica (**Grimont** & **Weill**, 2007; **Ranieri** *et al.,* 2013). There are approximately 2,600 non-typhoidal *Salmonella* serotypes (**Guibourdenche** *et al.,* 2010), nevertheless, over 60 % of human *Salmonella spp.* infections are due to *S. enterica* subsp. *enterica* (**CrumCianflone**, 2008) and almost 60% belong to *S. enterica* subsp. *enterica* (**Grimont** and **Weill**, 2007). *Salmonella* is a Gram-negative bacillus, rod shaped, non-sporeforming as shown in **Fig.** 1.1. Most *Salmonella* spp. are motile due to the presence of flagella around the cell

body (diameter ranges from 0.7 to 1.5 µm, length from 2 to 5 µm), except the avian-specific strains, S. *gallinarum* and S. *pullorum* (**Herman** *et al.,* 2008; **Foley** *et al.,* 2011, **Kim** *et al.,* 2014). They are considered as facultative anaerobic organisms and do not require oxygen for growth (**Jay** *et al.,* 2003). Strains of *Salmonella* can be differentiated serologically to various serovars based on the immune-reactivity of the two surface antigen groups, the O antigens (somatic antigens) and the H antigens (flagellar antigens) as well as capsular antigens (**Ranieri** *et al.,* 2013).

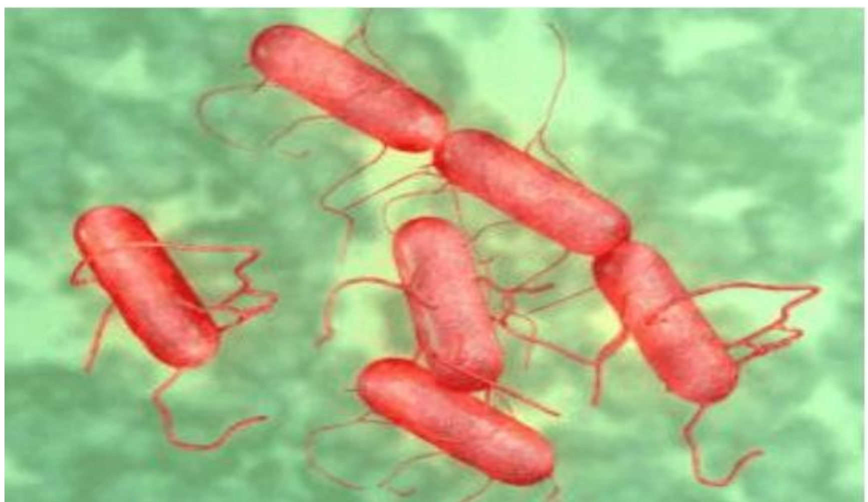


Fig. 1.1: The morphological shape of *Salmonella* bacteria (CDC, 2013).

Salmonella spp. are bacterial zoonotic pathogens and ubiquitous in the environment. However, they are commonly found in the gut and intestinal tract of animals including farmed, domestic and wild animals, therefore, there are numerous routes that facilitate their entry into the food chain (**Herman** *et al.,* 2008). Transfer of *Salmonella* to humans usually occurs by ingesting foods that are directly contaminated or cross-contaminated by animal faeces (**Modaressi** & **Thong**, 2010). They can grow in food stored at temperatures between 2–4°C and 54°C

(**Balamurugan**, 2010). Another study stated the range of the temperature was between 5.2°C to 46.2° C, with the optimal temperature being 35–43°C (**ICMSF**, 1996). *Salmonella spp*. have the ability to survive long term frozen storage (**Jay** *et al.,* 2003), for example, *Salmonella* was able to survive on frozen mangoes stored at -20°C for around 180 days (**Strawn** & **Danyluk**, 2010). Their ability to resist heat is dependent on several factors such as the composition, pH and water activity of the food (**Podolak** *et al.*, 2010).

Poultry and eggs are reservoirs from which *Salmonella* is passed through the food chain and ultimately transmitted to humans (**Finstad** *et al.,* 2012; **Howard** *et al.,* 2012). Most *Salmonella enterica* serotypes have the ability to colonize the ovaries and intestines of live poultry, and are commonly associated with raw poultry, eggs and other animal meats, as well as on the outside of the animal via the faecal contamination of hides, fleece, skin and feathers (**Howard** *et al.*, 2012; **Ricke** *et al.,* 2013). Consequently, cases of human salmonellosis are often as a result of the consumption of contaminated egg and poultry products (**Zaki** *et al.,* 2009). It has been reported that about 90-95% of non-typhoid salmonellosis is due to the consumption of contaminated food-stuffs (poultry and other meat products) (**Andreoletti** *et al.,* 2008). *Salmonella* serotype infection causes foodborne illness, microbial food spoilage and contamination of food products, leading to significant negative impacts on the economy (**Park** *et al.,* 2014). Thus, salmonellosis is a common public health concern, with an estimated 93.8 million cases of gastroenteritis, particularly in Europe, the United States, South America and Asia (**Rane**, 2011; **Borges** *et al.*, 2013). *S. entirica serovar enteritidis*

and S. entirica serovar typhimurium are typical serovars belonging to the *S. enterica* subspecies (79.9% of all known serovars in human cases) and are significant causes of foodborne illness in humans, with 95,548 reported cases in the European Union in 2011 (**Zweifel** & **Stephan**, 2012; **Ahmed** *et al.,* 2014). In general, S. entirica serovar enteritidis is reported more frequently than *S. entirica serovar typhimurium* in many European countries, but both are currently the most widely spread critical pathogens causing foodborne illnesses in humans and animals (**Park** *et al.,* 2014). Non-typhoid salmonellosis is a selflimiting gastroenteritis in humans and its symptoms include diarrhoea, stomach cramps, headaches, fever, abdominal pain, vomiting and nausea (**Herman** *et al.,* 2008) that lead to dehydration, weakness, and loss of appetite (**Kim** *et al.,* 2014).

1.2.2. *Campylobacter* spp

The genus *Campylobacter* belongs to the family *Campylobacteraceae* and currently consists of 25 species including *Campylobacter jejuni, Campylobacter fetus, Campylobacter coli, Campylobacter lari, Campylobacter upsaliensis, Campylobacter mucosalis, Campylobacter concicus, Campylobacter cuvus Campylobacter curvus, Campylobacter hyointestinalis, Campylobacter sputorum, Campylobacter rectus, Campylobacter gracilis, Campylobacter hominis, Campylobacter insulaenigrae, Campylobacter lanienae, Campylobacter laridis and Campylobacter showae* (**Man**, 2011). Nevertheless, *C. jejuni, C. coli*, *C. lari, C. fetus, C. hyointestinalis and C. upsaliensis* are the main causes of the human disease (**Lynch** *et al.,* 2012; **Taboada** *et al.,* 2013). *C. jejuni and*

C. coli are well recognized as the leading cause of bacterial foodborne diseases in both developed and developing countries (**Gilliss** *et al.,* 2013). For instance, between 80–90% of the infections in the US are caused by *C. jejuni* (**Kirkpatrick** & **Tribble**, 2011; **Taboada** *et al.,* 2013; **Gharst** *et al.,* 2013; **Vondrakova** *et al.,* 2014; **Fontanot** *et al.,* 2014). *C. jejuni* is widely present in poultry meat and slaughterhouses, especially raw and undercooked chicken (**Xu** *et al.,* 2013). *Campylobacter spp.* are Gram-negative, motile (cork-screw), non-spore-forming and S-shaped or spiral shaped (0.2–0.8 µm wide and 0.5–5 µm long), with a single polar flagellum at one or two in both ends as shown in the **Fig.** 1.2. Most *Campylobacter* species are strictly micro-aerophilic, requiring micro-aerobic conditions (5% oxygen, 10% carbon dioxide and 85% nitrogen) but some strains also grow aerobically or anaerobically (**Guyard-Nicodème** *et al.,* 2013). *Campylobacter spp* grow well between 37˚C and 42˚C, however, they cannot exist below 30˚C due to their lack of cold shock protein genes (**Levin**, 2007; **Velusamy** *et al.,* 2010). An infectious dose of *Campylobacter* is 10^6 cells (**Steele** & **McDermott**, 1984). Campylobacter also considered as the most common factor for Guillain-Barre syndrome.

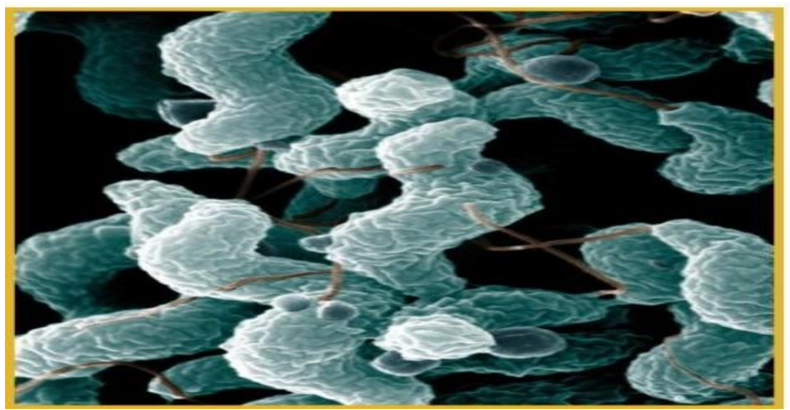


Fig. 1.2: Shape of campylobacter spp., Centre for Food Security and Public Health, Iowa State University, (2012)

Campylobacteriosis is a widely found prevalent foodborne illness in industrialized countries and was reported as the third most frequent bacterial foodborne disease. It causes diarrhoea, affecting an estimated 2.4 million people each year, representing about 0.8% of the population in the US (**Scallan** *et al.,* 2011). The symptoms of Campylobacteriosis present after an incubation period of between 24 and 72 hours, and include abdominal cramping and pain often associated with headaches, fever and vomiting, followed by diarrhoea (**Allos**, 2001; **Gharst** *et al.,* 2013). The consumption of undercooked poultry, pork and beef are considered major risk factors for sporadic infections of Campylobacteriosis (**Friedman** *et al.,* 2004; **Singh** *et al.,* 2011; **Fontanot** *et al.,* 2014).

1.2.3. *Staphylococcus* spp

Staphylococcus spp. are opportunistic Gram-positive bacteria, medically responsible for serious infections such as skin and wound infections in humans and animals (bovine mastitis) (**Chibli** *et al.,* 2014; **Peedel** &

Rinken, 2014). There are 32 species of these bacteria such *as Staphylococcus aureus, Staphylococcus intermedius, Staphylococcus hyicus and Staphylococcus epidermidis* (**Harris** *et al.,* 2002). The main strain is *S. aureus* which belongs to the genus of *Staphylococcus* and can be distinguished from other species, like *S. Epidermidis,* by the production of coagulase and thermo-nuclease enzymes (**Stehulak**, 2011). *S. aureus* is often responsible for postoperative infections and is easily transmitted upon contact (**Adak** *et al.,* 2013). However, other species of staphylococci such as the coagulase-negative *S. epidermidis*, are less likely to cause diseases in healthy subjects, but can infect implanted devices and catheters (**Chibli** *et al.,* 2014). Furthermore, S. *aureus* is a common cause of foodborne diseases. It is one of the top five pathogens that contribute to most foodborne illnesses in America, reported to be about 76 million cases of illness with 5000 deaths each year according to the Centre for Disease Control and Prevention (2012) (**World Health Organization**, 2007; **Sung** *et al.,* 2013).

S. *aureus* is non-spore forming, non-motile, catalase-positive organism as shown in **Fig.** 1.3 and is oxidase-negative, resistant to heat and antibiotics and can grow aerobically or anaerobically by aerobic respiration or fermentation. It can also grow at low water activity (approx. 0.86), with high concentrations of salt of approximately 14%. Generally, it requires an organic source of nitrogen, supplied by 5 to 12 essential amino acids such as arginine, valine, and B vitamins, including thiamine and nicotinamide (**Wilkinson**, 1997; **Lowy**, 1998; **Harris** *et al.,* 2002). *S. aureus* forms large and yellow colonies (**Harris** *et al.* 2002) and was so named

due to their golden colour. It has the ability to grow in a range of temperatures between 7 to 48.5°C, with an optimal temperature of 30 to 37°C, pH in the range of 4.2 to 9.3, the optimum being 7 to 7.5 and sodium chloride (NaCl) concentrations up to 15% (**Bhatia** & **Zahoor**, 2007).

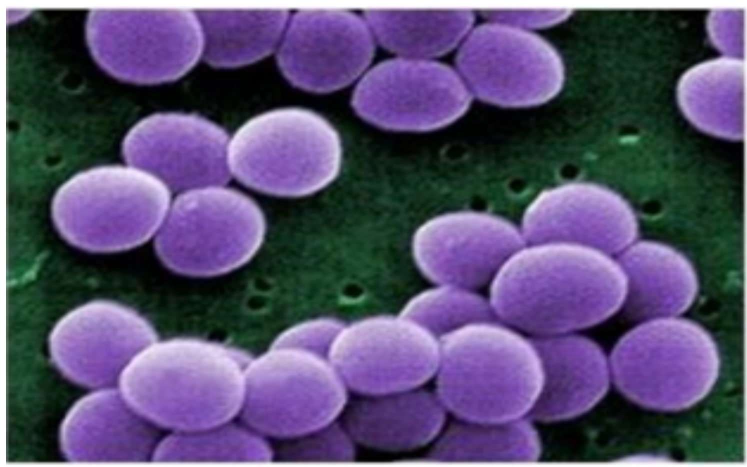


Fig. 1.3: The morphological shape of *Staphylococcus aureus*

S. aureus enterotoxins (SEs) are proteins that have extracellular thermostability and ingestion of food contaminated with SEs causes staphylococcal food poisoning (SFP) and is the second most commonly reported foodborne illness (**Argudin** *et al.,* 2010). The symptoms are usually observed within a few hours of eating the contaminated food and include nausea, vomiting, abdominal cramping, and prostration. Food which is frequently infected by S. *aureus* includes meat and meat products as well as poultry and egg products (**Stehulak**, 2011).

1.2.4. *Escherichia coli* (0157:H7)

Escherichia coli was first discovered by the German bacteriologist Theodor Escherich and is the most common gastrointestinal tract of

humans and animals and environmental contaminant. Most *E. coli* strains are harmless, but some, such as serotype 0157:H7, are pathogenic, causing severe intestinal and extraintestinal diseases (food poisoning) in humans (**Leimbach** *et al*., 2013; **Kaper** *et al.,* 2004). It is considered as one of the most serious foodborne pathogens causing serious complications (**Fedio** *et al.,* 2011; **Blount**, 2015). *E coli* strains are common examples of coliform bacteria usually present in the environment and in warm-blooded organisms such as human and animals (**Rompre** *et al.,* 2002). They are Gram-negative bacilli, non-spore forming, rod shaped bacteria, approximately 2.0 µm long and 0.25-1.0 µm in diameter, with a cell volume of 0.6–0.7 µm as shown in **Fig.** 1.4. They belong to the *Enterobacteriaceae* family and they may or may not be mobile because some rods are flagellated and some are not. They are facultative anaerobes and ferment simple sugars such as glucose to form lactic, acetic, and formic acids (**Scheutz** *et al.,* 2011).

Usually, *E. coli* are found as commensals in the gastrointestinal tract of many animal species and their presence in faeces indicates the consumption of contaminated food samples (**Singh** & **Saxena**, 2016). Their ability to survive outside the body for a long time makes them an ideal indicator organism to test food and environmental samples for faecal contamination (**Samuel** *et al.*, 2011). *E. coli* may grow on a solid or in a liquid growth medium under optimal laboratory conditions (temperature is 36.7°C, with a range of 45 to 45.6°C, pH of 6.0 to 8.0). It can grow in a pH as low as 4.3 and as high as 9 to 10 (**Mitscherlich** & **Marth**, 1984). After ingestion, illness can occur between 8 and 44 hours of an infective dose of approximately 10^8 to 10^{10} microorganisms. The

natural reservoirs of this pathogen occur in many types of animals, such as cattle (ground beef), sheep, goats, and wild animals (**Doyle** & **Schoeni**, 1984). Poultry based food, especially chicken, is most likely reservoir for *E. coli* serotype 0157:H7 (**Ferens** *et al.,* 2011; **Bergeron** *et al.*, **2012)**. In addition, it can contaminate food produced, stored and/or marketed under unhygienic conditions (**Zafar** *et al.,* 2016). The serological character of *E. coli* is determined by the O antigens on the bacterial surface, these antigens are lipopolysaccharides and their polysaccharide moiety plays a key role in serological specificity (**Jann** *et al.,* 1971).

There are four strains or categories that cause diarrheal illnesses or disease, enteropathogenic *E. coli*, enter-invasive *E. coli*, enterotoxigenic *E. coli* and enterohemorrhagic *E. coli.* Pathogenic *E. coli* have been classified into different types that cause this common disease by using an assortment of virulence factors (**Kaper** *et al.,* 2004). Some *E. coli* strains such as serotype (EHEC) 0157:H7 are pathogenic and have great potential in causing health problems to people, such as food poisoning, which are a global concern (**Rahal** *et al.,* 2012). It was first recognized as a cause of illness in 1982, when it caused two outbreaks of haemorrhagic colitis traced to the consumption of hamburgers in the USA (**Dincoglu** *et al.,* 2016).

Fig. 1.4: The morphological shape *of E. coli* (adapted from Uyen Nguyen,2015).

1.2.5. *Norovirus*

Viruses of the genus *Norovirus* are one of the most well-known viruses causing foodborne diseases, and are responsible for 45% of recreational waterborne outbreaks, followed by members of the genus *Adenovirus* which are responsible for 24% (**Sinclair** *et al.,* 2009; **LaRosa** *et al.,* 2012). *Norovirus* (previously denoted as "Norwalk-like viruses") was first recognized in Ohio in 1968, during the outbreak of a winter vomiting disease (**Adler** & **Zickl**, 1969; **Patel** *et al.,* 2009). As reported in **Marshall** and **Bruggink** (2011), *Norovirus* (NoV) belongs to a genetically diverse group of non-enveloped, single stranded RNA viruses of the family *Caliciviridae*. This group is currently subdivided into five subgroups, *Norovirus, Sapovirus, Lagovirus, Vesivirus and Nebovirus*, and one or more species is recognized in each genus. Noroviruses are responsible for acute gastroenteritis outbreaks around the world. In the USA, there are 5.5 million annual cases of foodborne *Norovirus* diseases (**Hall** *et al.*, 2012) and it was the second most reported cause of foodborne outbreaks in the UK in 2007–2008 (**Wheeler** *et al.*, 2005; **Baert** *et al.,* 2008).

Noroviruses are resistant to many disinfectants, so remain infectious for about two weeks on surfaces and for more than two months in water (**Seitz** *et al.,* 2011; **Park** *et al.,* 2011). There are many ways by which *Norovirus* can spread, including the direct transmission from one person to another, by faecal contamination and the ingestion of aerosolized vomitus. It can also be transmitted indirectly via contaminated surfaces, water or food (**Hall** *et al.,* 2012. Noroviruses are small (27–32 nm) non-enveloped viruses with a linear, positive-sense and single stranded RNA

genome (**Green**, 2007) as shown in the **Fig.** 1.5). The single stranded RNA genome of human *Norovirus*es (hNoVs) is approximately 7600 nucleotides (nts) in length and protected within a virus capsid composed of 90 dimeric copies of a 60 kD protein VP1, and a basic minor protein component (VP2) involved in the expression and stability of VP1 (**Chen** *et al.,* 2007; **Knight** *et al.,* 2012). *Noroviruses* can remain unaffected by pH (2– 9), a temperature of 60°C for 30 min and in cold temperatures, they can survive for years (**Cannon** *et al.*, 2006), while they are destroyed by rapid boiling (**Summa** *et al.,* 2012).

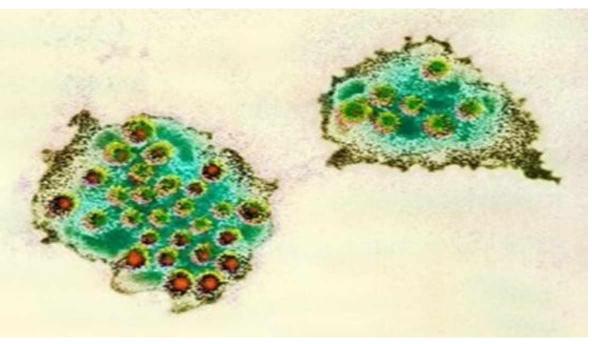


Fig. 1.5: Shape of norovirus (adapted from Marler Clark ,2013).

1.3. Detection methods

1.3.1. Conventional techniques for the detection of pathogens

Conventional culture methods are still the most reliable and accurate techniques for the detection of foodborne diseases as illustrated in the **Fig.** 1.6 (**Velusamy** *et al.,* 2010). These techniques include microscopy (a simple technique and easy to use), culture (the gold standard) and serology (the mainstay of diagnosis for many diseases). Although these are inexpensive and protracted methods, there are disadvantages in each method, for example, microscopy has limited sensitivity, cultivation

runs the risk of contamination with commensal flora and the possibility of reduced viability during transportation. The main disadvantages of serology are its requirement of convalescent sera (serum obtained from one who has recovered from an infectious disease and considered to be especially rich in antibodies against the infectious agent of the disease) and the occurrence of false positive results due to cross reactivity with other organisms (**Lim** *et al.,* 2005; **Francy** *et al.,* 2009). In addition, some standard methods for the detection of *Listeria monocytogenes* may need up to 7 days to obtain results (**Artault** *et al.*, 2001). Other viable bacteria in the environment can enter a dormant state, where they become non-culturable, viable but non-culturable (VBNC), which can subsequently lead to an under estimation of pathogen numbers or a failure to isolate a pathogen (**Toze**, 1999; **Velusamy** *et al.*, 2010).

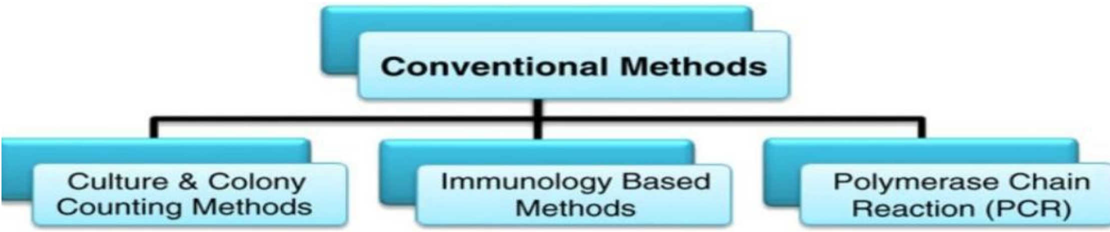


Fig. 1.6: Conventional methods employed for pathogen detection (adapted from Velusamy *et al.,* 2010).

1.3.2. Rapid methods

Conventional culture based methods for identification of bacterial pathogenic agents are widespread in the food industry (**Velusamy** *et al.,* 2010; **Ishikawa** *et al.,* 2014). Rapid detection of pathogenic organisms that cause foodborne diseases is needed to ensure food safety (**Liu** *et al.*, 2012; **Bakthavathsalam** *et al.*, 2013) because it is more suitable for

quality assurance management systems applied in food manufacturing processes. Many studies are focussed on developing rapid methods for foodborne pathogens and toxins with different aspects of detection such as sensitivity, rapidity, selectivity and those with the potential for on-site analysis. These rapid detection methods can be classified into three categories: nucleicacid-based molecular methods, immunological methods, and biosensors that depend on immunoassays and molecular techniques (**Zhao** *et al.*, 2014).

1.3.2.1. Steps of rapid detection

Generally, there are two significant steps for application of rapid technologies: firstly, the fishing/pre-concentration method (responsible for the selectivity), in which the microbial and viral species or group of interest is removed and either tagged or amplified to differentiate it from the remaining material in the sample.

Secondly, the detection of an analyte by counting and quantitative measurement using a detector which typically acts as a transducer, translating the biological, physical, or chemical alteration into a measurable signal (**Noble** & **Weisberg**, 2005).

1.3.2.2. Fishing/pre-concentration methods

Capture methods used in rapid microbial detection technology can be grouped into three broad areas. The first method is the molecular surface recognition method which captures and/or labels the target microorganism by binding to molecular structures on the exterior surface or to structures within the interior of a bacterium, virus, or to the genetic

material of interest. These methods include immunoassay techniques, bacteriophage, and molecule-specific probes, such as lipid or protein attachment-based approaches (**Noble** & **Weisberg**, 2005). Secondly, nucleic acid detection methods target specific nucleic acid sequences of bacteria, viruses, or protozoa. These techniques include PCR, reverse transcriptase polymerase chain reaction (RT-PCR), quantitative PCR (QPCR), and nucleic acid sequence based amplification (NASBA) (**Noble** & **Weisberg**, 2005). The third method is enzyme/substrate methods which depend on either the existing chromogenic or fluorogenic substrate methods which are already widely used, or new enzyme-substrate approaches. Several new technologies are in development using this technology in conjunction with high sensitivity fluorescence detection instruments to reduce the time required for the assay (**Noble** & **Weisberg**, 2005).

Molecular recognition approaches are more rapid, sensitive and adaptable to a wider range of indicators and pathogens. However, antibody (Ab)-based approaches have a significant advantage of the specific binding affinities between the Abs and specific antigens which can be produced in the laboratory and purchased commercially (**Kooser** *et al.,* 2003). The Abs are specifically applied for a single strain of any bacteria, such as *E. coli* O157:H7*,* as they can be potentially produced for single species, groups or families of organisms such as enterococci hence, the latter two approaches are generally more difficult (**Noble** & **Weisberg**, 2005).

1.3.2.3. Detection techniques

Capture methods can generate chemical, optical and biological signals that are detected by various detection technologies which are dependent on the measurement of optical, electrochemical, or piezoelectric properties (**Deisingh**, 2003). The most common detection methods are optical techniques that use detection units, such as spectrometers and fluorimeters, which can be used for spectroscopic or fluorescent detection of bacteria. Additionally, another frequently used method is flow cytometry (FCM), which is based on characteristics such as natural fluorescence or light scattering (**Veal** *et al.,* 2000). Other options for optical detection include fibre optics and laser-based interferometry, evanescent wave-based technologies which allow the measurement of bound fluorescently labelled antibodies to antigens on the fibre surface (**Noble** & **Weisberg**, 2005). Electrochemical detection methods are fast, but they require special equipment for measuring the signal. Electrochemical techniques are not as susceptible to turbidity interference as optical-based detection and typically have very low detection limits. Much research has focussed on the development of novel electrochemical applications to measure bacteria (**Perez** *et al.,* 2001).

Currently, piezoelectric detection methods are most commonly paired with Abantigen capture modes. Microorganisms captured by specific antibodies are immobilized onto the surface of the quartz crystal, which is then subjected to an electrical field. Piezoelectric biosensors have been used to detect *S. entirica serovar typhimurium* and *L. monocytogenes* in

food (**Babacan** *et al.,* 2002; **Vaughan** *et al.,* 2001). Immunoassay methods have also been adapted from technologies applied for the rapid detection of food borne pathogens (**Lim**, 2001). Some workers developed an innovative method to detect pathogens such as *E. coli* O157:H7 directly from different food (**Demarco** & **Lim**, 2001, **DeMarco** & **Lim** 2002; **Kramer** *et al.*, 2002). Biotin-streptavidin interactions were used to attach polyclonal anti-*E. coli* O157:H7 antibodies to the surface of a fibre optic probe (**Noble** & **Weisberg**, 2005).

Any detection method which uses a chemical, optical or biological signal is generally referred as a rapid method. Such rapid methods, including antibody or nucleic acid-based assays, are modified or improved compared to conventional techniques (**Doyle** & **Beuchat**, 2013). They are of high value for food industries by providing significant advantages such as speed, specificity, sensitivity, cost and labour efficiency (**Park** *et al.,* 2014).

New advanced molecular and immunological methods need just a few hours for detection of the targeted pathogen from food samples compared to 4-5 days using conventional culture based methods (**Hadjinicolaou** *et al.,* 2009). To increase the sensitivity of the rapid method, it is necessary to employ nonselective or selective enrichment steps. However, it should be noted that this could increase the total assay time (**Ukeda** & **Kuwabara**, 2009; **Mihayara** *et al.,* 2010). According to the Food and Drug Administration (**FDA**), any rapid detection method that indicates the presence of the target foodborne pathogen (positive

results) must be confirmed by traditional culture based methods (**FDA**, 2001; **Park** *et al.,* 2014).

1.4. Immunoassay techniques

1.4.1 Introduction

Immunoassays (IA) utilize the binding reaction of target substances (antigen) with an antibody to produce a signal which can be measured (**Fingerová**, 2011). **Landsteiner** (1945) was the first researcher to report the concept of an immunoassay, revealing that an antibody could selectively bind to a small molecule. Subsequently, the immunoassay was reported at the end of 1950s by **Yalow** and **Berson** (1960), who developed a radioimmunoassay during their study of insulin metabolism. This technology was first applied in agriculture in 1970, when **Centeno** and **Johnson** (1970) developed antibodies that specifically bound to the insecticides, DDT and Malathion. The immunoassay has been widely used for food toxins, such as mycotoxins in grain (**Casale** *et al.,* 1988), as well as pathogens (**Webster** *et al.,* 2004).

In nature, numerous antibodies are produced by the immune systems of animals at any given time (**Sheedy** & **Yau**, 2011). Measuring analytes in an immunoassay is achieved in two main ways, either competitive or non-competitive. In a competitive immunoassay, the amount of unlabelled analyte (antigen) in the test sample is measured by its ability to compete with labelled antigen to bind antibody, where the unlabelled antigen decreases the ability of the labelled antigen to bind the antibody due to the binding site being already occupied. So, in the competitive

immunoassay format, a small amount of the measured labelled antigen in the sample means there is more unlabelled antigen (analyte), which can be expressed by the inverse relationship between the response signal and amount of analyte in the sample. In a non-competitive immunoassay, also referred to as a sandwich assay, the analyte is bound (sandwiched) between two highly specific antibody reagents. This type of assay generally provides the highest level of sensitivity and specificity, and is applied for the measurement of a critical analyte. The non-competitive assay format comprises one or two steps like the competitive assay. In a non-competitive immunoassay, the amount of measured antigen in the sample is directly proportional to the response signal, which means that more antigen gives stronger response signals.

1.4.2 Homogeneous and heterogeneous immunoassays

Immunoassay methods can be classified into homogeneous and heterogeneous according to their need for separation of the complex formed by binding between labelled antibody and target antigen. Methods which require separation are referred to as a heterogeneous assay, whereas, methods in which separation is unnecessary are referred to as homogeneous assays as illustrated in **Fig.** 1.7. Homogeneous assays are generally easier and usually applied to measure small-sized analytes like therapeutic drugs (**Fingerová** *et al.,* 2011).

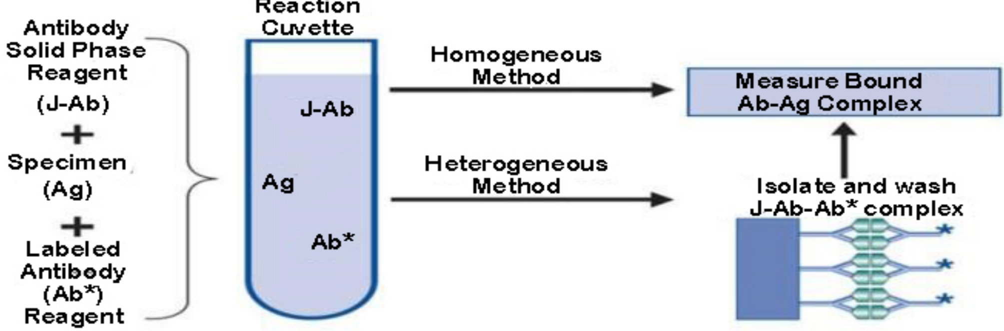


Fig. 1.7: Schematic diagram illustrating difference between homogeneous and heterogeneous immunoassays (Fingerová *et al.*, 2011**).**

1.4.3. Enzyme-linked immune sorbent assay (ELISA)

The enzyme-linked immune sorbent assay (ELISA) is a biochemical technique that combines an immunoassay with an enzymatic assay into one method, where an enzyme linked to an antibody is used as a marker for the detection of different analytes (**Jasson** *et al.,* 2010). The ELISA is one of the most widely methods for foodborne pathogens, as it is very accurate and sensitive method for detecting antigens or haptens (small molecules that can elicit an immune response only when attached to a large carrier such as a protein) (**Chen** & **Lin**, 2007; **Mandal** *et al.*, 2011).

The detection of an analyte using ELISA can be achieved in both liquid reagent (wet lab) and by dry strips (dry lab) (**Cohen** & **Kerdahi**, 1996). Despite its advantages in comparison with conventional culture based methods, the sensitivities of commercial ELISAs are widely different depending on sampling times and processing methods, in addition to the possibility of giving false negative results (**Park** *et al.,* 2014). Typical ELISAs include various steps such as blocking, washing, incubation of primary and secondary antibodies and substrate development (**Park** *et*

al., 2014). Analysis using ELISA usually takes from one to several hours, making it an inadequate for the rapid and real-time determination of analytes (**Ramírez** *et al.*, 2009). In contrast to the nucleic acidbased assays which require good technical expertise and an extraction step, the antibody-based measures, such as immunobiosensor or ELISA and immunechromatographic strip, can be applied in the field with little technical knowledge or instruments and high-volume testing (**Feng** *et al.,* 2014).

New approaches for the development of ELISA techniques utilizes nanotechnology to improve sensitivity and reduce the time required to achieve detection. A functional nanoparticle enhanced enzyme-linked immunosorbent assay (FNPELISA) used immunomagnetic nanoparticles (IMMPs) conjugated with a monoclonal anti-O157:H7 antibody to capture *E. coli* O157:H7 (**Shen** *et al.,* 2014). Nanotechnology has also been used to develop a novel universal reagent for immunoassays. This reagent can be used for the simultaneous detection of pathogenic bacteria like *E. coli* O157:H7, *Salmonella spp*. and *L. monocytogenes* in food (**Chen** *et al.,* 2006).

1.4.4. Lateral flow immunoassay (LFI)

Lateral flow immunoassay (LMI) is one of the rapid methods being used in bacterial detection; it is referred to as the immunochromatographic assay (**Blažková** *et al.,* 2009). It is a simple device intended to detect the presence or absence of a target analyte in a sample (matrix) without the need for specialized and costly equipment (**Posthuma-Trumpie** *et al.,* 2009). The technical basis of LMI was derived from the latex agglutination

assay (**Wong** & **Tse**, 2009). Lateral flow devices (LFD) typically comprised a simple dipstick made of a porous membrane that contains coloured latex beads or colloidal gold particles coated with specific antibodies targeted against a microorganism (**Betts** & **Blackburn**, 2009). The LMI technique is simple to use and easy to interpret, and does not require any washing or a manipulation. It can also be completed within 10 min after culture enrichment (**Aldus** *et al.,* 2003). There are various LFDs on the market that have been validated for detecting different foodborne pathogens (**Jasson** *et al.,* 2010). Lateral flow assay is designed to comprise a variety of materials, each of which can perform one or more function, and all the assembly parts are mounted on a backing card that forms the assay kit or strip. These parts comprise a sample pad, conjugate pad, reaction matrix and absorbent pad arranged in a horizontal position, where the sample passes through them from the sample pad reaching the absorbent pad or wick as shown in the **Fig.** 1.8 (**Wong** & **Tse**, 2009).

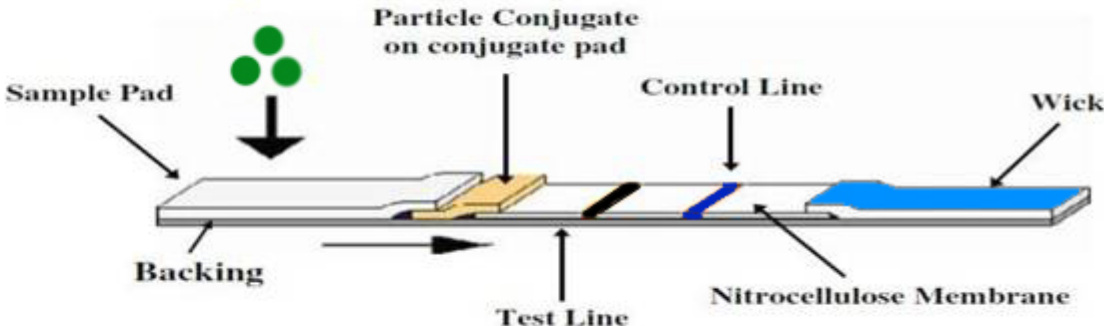


Fig. 1.8: Schematic of configuration of lateral flow parts cited (Wong and Tse, 2009).

The analysis starts when the sample is added to the proximal end of the strip (sample pad). In this area of the strip, the sample is treated to become compatible with the next stage of analysis. Then, the treated sample migrates through this region to the conjugate pad, where the

particulate conjugate is immobilized. This particulate may be colloidal gold or florescent or other coloured particles, and these conjugated particulates bind to the biological molecules that can be an antigen or antibody according to the assay format. The analyte in the sample interacts with biological recognition receptor conjugated with gold nanoparticles, and migrate together towards a porous nitrocellulose membrane onto which another immobilized biological recognition receptors. These biological molecules are either antigens or antibody laid down in bands of specific areas in the porous membrane, where they capture the analyte and the conjugate as they migrate through the capture lines. Finally, excess reagent moves past the capture lines to be trapped in the absorbent pad, which the last stage of the strip. Reading of the results can be achieved using the naked eye or by readers (**Wong** & **Tse**, 2009).

Lateral flow is one of the most appropriate techniques to be applied in the field of point-of-care analysis, being used for medical diagnostics, therapeutic purposes, environment, industry, food safety, agriculture and animal health. Its advantages include ease of manufacture, stability, ease of use due to minimal operator experience and it is considered as a low cost method which gives realtime results. However, it has some disadvantages relating to sensitivity, test to test reproducibility, in addition to some limitations in quantitative analysis (**Wong** & **Tse**, 2009). Many studies have used and developed the immunoassaybased lateral flow dipstick for the rapid detection of organisms and enterotoxins, such as **Jung** *et al.* (2005) who developed a colloidal immunochromatographic strip for the detection of *E. coli* O157:H7 in enriched samples. They

reported a minimum detection limit of 1.8×10^5 CFU/ml without enrichment and 1.8 CFU/ml after enrichment. Another study detected aflatoxin B1 in pig feed, with a visual detection limit for aflatoxin B1 of 5 μg/kg (**Delmulle** *et al.,* 2005).

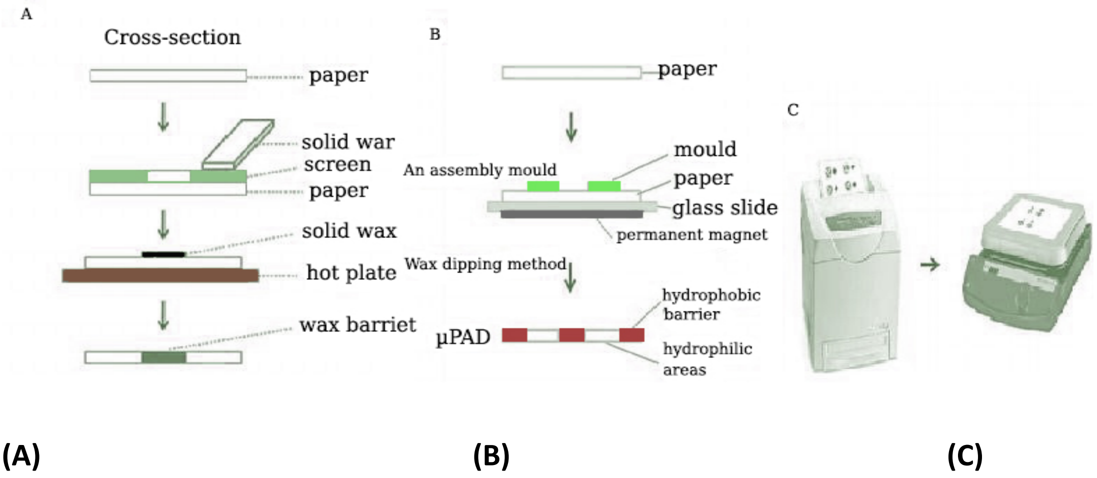
1.4.5. Paper-based microfluidic immunoassay (MI)

In paper-based microfluidic methods, paper strips are used to transport liquid samples. These paper strips are impregnated with a hydrophobic material, such as paraffin wax, to prevent cross contamination between test zones and to prevent spreading of the sample over a large area enabling good uniformity and estimation of concentration through the colour intensity. Paraffin wax is preferred in order to form water repellent zones in papers and strips because of its inertness to chemical reagents and suitability to make different patterns (**Yetisen** *et al.,* 2013).

Paper as a solid phase material provides three main requirements to be a comparable material to be used in diagnostic methods, processing biological samples using a small volume, short time duration to give test result and should provide good binding sites for proteins to enable forming sharp capture lines.

Fabrication techniques of paper-based fluidic devices can be made in 2 or 3 dimensions to transport fluids in horizontal and vertical directions depending on the complexity of the diagnostic devices. The construction of wax barriers is a critical step in the fabrication of paper-based microfluidics and can be achieved in different ways including (1) screen printing in which a wax player is spread on the surface of paper sheet,

then placed on a hot plate or an oven to melt and diffuse the wax into the paper. (2) Wax dipping, in this method an iron mould is used to form a pattern of wax on the paper, where it is fixed on the paper making an assembly that is rinsed in a molten wax (**Songjaroen** *et al.,* 2011). These two modes of wax patterning suffer from inflexibility and low reproducibility between batches. (3) Wax printing, in this modern technique a sheet of paper is used for designing and impregnating of wax and using wax printer, the waximpregnated paper sheet is subjected to heat either by a hot plate or oven to fix wax barriers (**Carrilho** *et al.,* 2009). These three types of wax patterning are illustrated in **Fig.** 1.9.



(A) **(B)** **(C)**

Fig 1.9. Patterning techniques of wax on paper sheets (A) represents screen printing technique, (B) represents dipping technique and (C) represents wax printing (Yetisen *et al.*, 2013).

1.4.6. Immunomagnetic separation assay (IMS)

Immunomagnetic separation (IMS) is a technique for the isolation of living cells, either prokaryotes or eukaryotes, from a test sample depending on an antigenantibody reaction. In this technique, super para-

magnetic particles or beads are coated with specific antibodies of target cells, then mixed with the liquid sample to capture the target analyte. Many detection methods can be used, involving cultivation methods and molecular methods or immunoassay methods using a second antibody (sandwich format) to determine the amount of analyte in the sample (**Olsvik** *et al.,* 1994), as the bound cells on the magnetic beads remain viable. IMS can utilize immunomagnetic beads (IMBs) as capturing reagents for the microbial isolation and identification of foodborne diseases (**Zhao** *et al.,* 2014). The IMS procedure consists of two fundamental steps; first, the target cells are mixed with immune-magnetic particles followed by incubation for less than one hour and separated by an appropriate magnetic separator; then, the magnetic complex is washed several times to remove the contaminants (**Mandal** *et al.,* 2011). There are several advantages of magnetic separation techniques in comparison with standard separation techniques, for example, IMS using antibody conjugated magnetic nanoparticles is a potential and well-established tool for the specific separation of bacterial contaminations from complex matrices (**Roda** *et al.,* 2012).

Binding between antibody, which is mounted on the magnetic bead, and target cells can be achieved by the methods shown in **Fig.** 1.10. First, the specific antibody fixed on the bead directly binds to the antigen; second, the specific antibody binds to the antigen via a second antibody, and the third method involves a specific antibody that binds firstly to the antigen, then to the magnetic bead (**Olsvik** *et al.,* 1994).

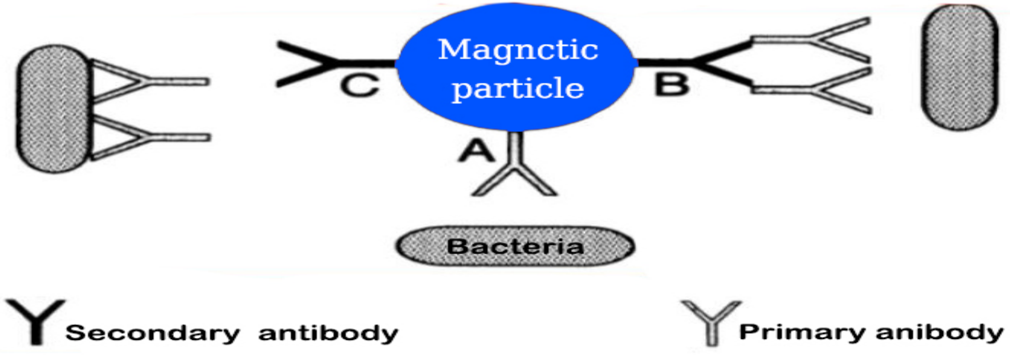


Fig. 1.10: Methods of antibody/antigen binding in immunomagnetic separation (adapted from Olsvik *et al.,* 1994).

1.4.7. Radioimmunoassay (RIA)

Radioimmunoassay (RIA) is an immunological technique which depends on the use of radioactive substances, such as H^3 and I^{125}, as a label for the antigen (**Brown** *et al.*, 2010) in an indirect immunoassay method, in which a limited amount of a specific antibody is used to quantify the target antigen. The principle of the RIA is based on the assumption that an antigen can bind to a radioactive molecule, retaining its ability to attach antibodies (**Abraham** *et al.,* 1971) in order to form an antigen-antibody complex that is chemically different either from the antigen or antibody itself.

Radioimmunoassay is one of the most frequently used methods in the analysis of hormones because of its high sensitivity and specificity, although people who use this technique must be licensed for the use of radio-isotopic tracers. The RIA is usually a competitive format in which a limited amount of specific antibody is combined with two types of antigen, labelled and unlabelled. In this format, the amount of labelled antigen/antibody complex detected is inversely proportional to the

amount of unlabelled antigen in the sample as illustrated in **Fig.** 1.11 (**Abraham** *et al.,* 1971).

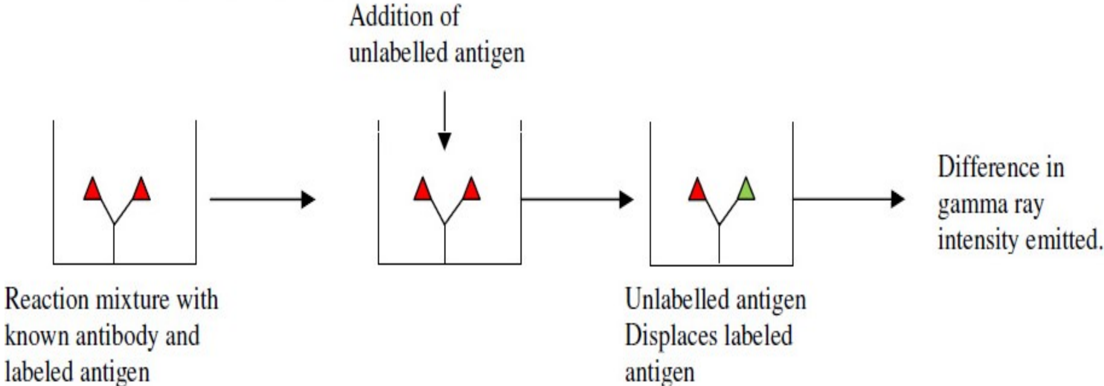


Fig. 1.11: Steps of indirect radioimmunoassay. A limited amount of specific antibody mixed with two types of antigen (labelled and unlabelled). (adapted from Hunter *et al.* 1978).

The mechanism of a competitive RIA depends on the ability of unlabelled antigen to block the binding sites on antibodies decreasing the probability of attachment between labelled antigen and antibodies, as shown in **Fig.** 1.12 below.

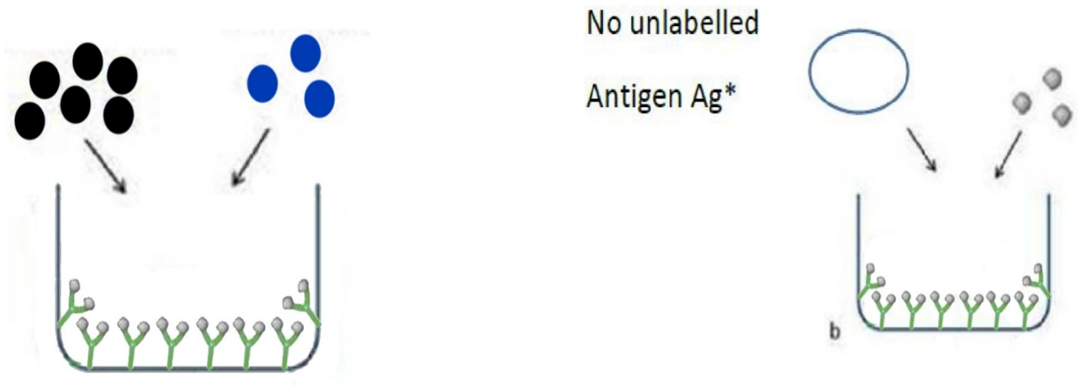


Fig. 1.12: Competitive radioimmunoassay: the unlabelled antigen limits the ability of the labelled antigen to complex with the antibody (Hunter *et al.* 1978).

1.4.7.1. Types of radioimmunoassay

1.4.7.1.1 A single antibody radioimmunoassay

In this process, the first antibody, which is specific for the target antigen, is incubated with labelled and unlabelled antigens, resulting in competition for binding to antibody between the two types of antigens (**Desbuquois** *et al.,* 1971). The unbound antigens are isolated by the adsorption to dextran-coated charcoal, in this way, only free antigen is removed because dextran blocks large sized pores on charcoal and prevents the antigen-antibody complex from being adsorbed due to its large size. After centrifugation, the antigen-antibody complexes are isolated in the supernatant as shown below in **Fig.** 1.13.

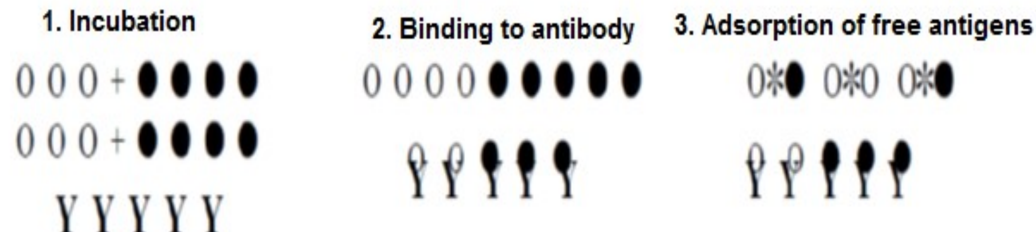


Fig. 1.13: Schematic of a single antibody RIA: clear circles refer to unlabelled antigen, black circles refer to labelled antigen, Y-shaped refers to antibody and stars refer to charcoal (adapted from Desbuquois *et al.,* 1971**).**

1.4.7.1.2. Double antibody RIA

In this format, the precipitated complex contains Ag-Ab1Ab2, that is, a second specific antibody for the immunoglobulin of the antigen is used (**Heding** *et al.,* 1971). The first step in this process is to incubate the first antibody with the labelled and unlabelled antigens (labelled tracer can be added either simultaneously with unlabelled or may be added after

incubation period, but the addition after the incubation period increases sensitivity). The second step includes the addition of a secondary antibody, after which precipitation will occur, sometimes polyethylene glycol is added to the reaction in order to decrease the solubility of the complex, thereby improving precipitation as illustrated in **Fig.** 1.14.

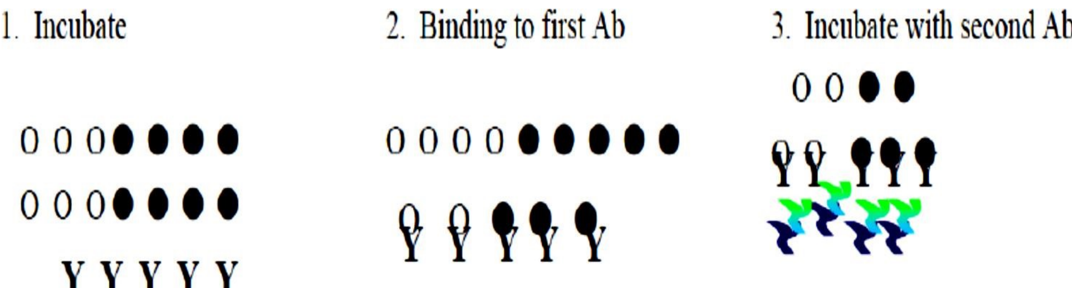
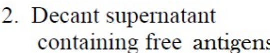


Fig. 1.14: Schematic of a double antibody RIA: the first step in this process is to incubate the first antibody with the labelled and unlabelled antigens, the second step includes the addition of a secondary antibody (adapted from Heding *et al.,* 1971).

1.4.7.1.3. Solid phase RIA

In solid phase RIA, a RIA specific antibody is fixed on a solid surface, like plastic assay tubes, then, the labelled and unlabelled antigens are added and incubated with the antibody (**Forghani** *et al.*, 1974). The unbound antigens are removed by decantation of the supernatant and there is no need for a centrifugation step, so it is a simpler assay than the other two types of RIA previously described as shown in **Fig.** 1.15.

1. Incubate with sample and tracer



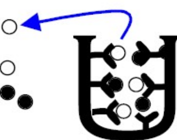


Fig. 1.15: Schematic of solid phase RAI (adapted from Forghani *et al.,* 1974).

1.4.8 Immunofluorescence (IF)

An immunofluorescent immunoassay is dependent on the antigen-antibody interaction in which the antibody is labelled with a fluorescent dye and then, the antigen-antibody complex is investigated by different methods such as ultraviolet microscopy. There are two basic techniques of immunofluorescence, direct immunofluorescence (DIF) and indirect immunofluorescence (IIF).

In the indirect format, there are two types of specific antibodies, one directed toward the antigen, known as the primary antibody as it is usually label-free, while, the other is labelled and directed to the primary antibody (secondary antibody) as shown in **Fig.** 1.16.

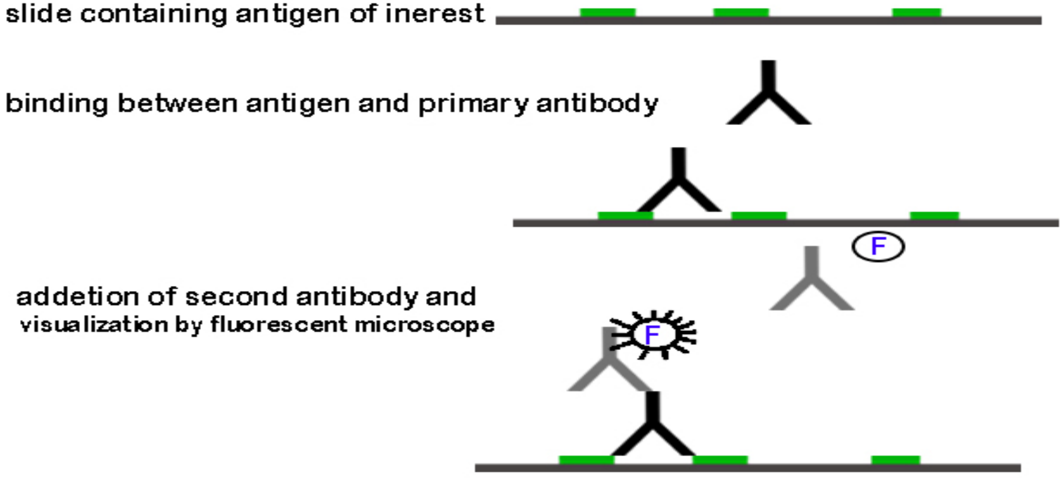


Fig. 1.16: Schematic of indirect fluorescence showing the two assay steps (adapted from Webster *et al.,* 2004**).**

In the direct assay format, there is only one type of labelled antibody which is directly attached to the target antigen. This format is less

sensitive in comparison with indirect immunofluorescence and the procedure is illustrated below in **Fig.** 1.17.

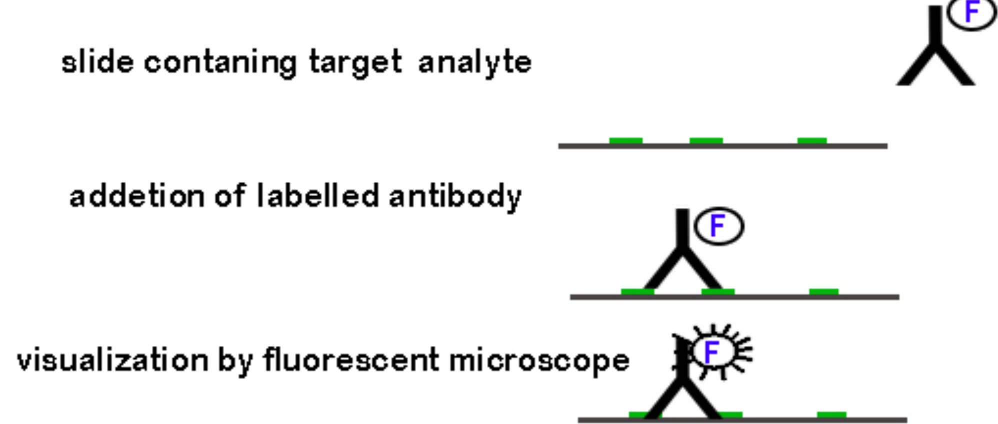


Fig. 1.17: Schematic of the direct immunofluorescence (adapted from Webster *et al.,* 2004).

1.5. Molecular techniques

Molecular detection systems are extensively used in food analysis for the detection of bacteria such as *Listeria spp*., *Salmonella spp*. and *E. coli* 0157:H7 (**Loff** *et al.,* 2014). The main advantage of nucleic-acid-based food pathogen detection assays is the high level of specificity, as they detect specific nucleic acid sequences in a target organism by hybridizing them to a short synthetic oligonucleotide complementary to a specific nucleic acid sequence. There are many DNA-based assay formats, but only probes and nucleic acid amplification techniques have been developed commercially for detecting foodborne pathogens (**Bisha** & **Brehm-Stecher**, 2010). These techniques are based on the detection of nucleic acids and they have significant benefits because DNA molecules show constant concentrations, stability, and better extraction than proteins (**Santiago-Felipe** *et al.,* 2014). Many types of nucleic-acid-based

assays, including amplification, hybridization, microarrays, and biochips, have been developed to be rapid methods for detecting foodborne pathogens (**Zhao** *et al.,* 2014). ISO standards have been established, providing guidelines to qualitatively detect foodborne pathogens by PCR, such as ISO 22174:2005, ISO/TS 20836:2005, ISO 20837:2006 and ISO 20838:2006 (**Falentin** *et al.,* 2010).

1.5.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a nucleic acid amplification technology that was developed in the 1980s (**Mullis** *et al.,* 1986). It is based on the isolation, amplification and quantification of a short DNA sequence from the genetic material of the targeted bacteria. Despite having resolved several problems in traditional detection methods (**Moreno** *et al.,* 2011), it is unable to distinguish between viable or dead cells, which could lead to false positive results (**Okoh** *et al.,* 2007; **Moreno** *et al.,* 2011). PCR is also less time consuming than other techniques, such as culturing and plating which takes between 5 to 24 h to produce a detectable result, but this depends on the specific PCR variation used and does not include any previous enrichment steps. PCR uses oligonucleotide primers usually 20–30 nucleotides in length, which are homologous to the ends of the genomic DNA region to be amplified. The process is performed in repeated cycles, so that products of one cycle serve as the DNA template for the next cycle, doubling the number of target DNA copies in each cycle (**Hill**, 1996).

A variety of PCR methods have been developed for bacterial detection, such as real-time PCR (**Rodrìguez-Lázaro** *et al.,* 2005), multiplex PCR

(**Jofrè** *et al.,* 2005) and reverse transcriptase PCR (RT-PCR) (**Deisingh**, 2004). Amongst the different PCR variants, multiplex PCR is very useful as it allows the simultaneous detection of several organisms by introducing different primers to amplify DNA regions coding for specific genes of each bacterial strain targeted (**Touron** *et al.,* 2005). Real-time PCR permits quicker results without much manipulation and depends on fluorescent emission to detect the amplified DNA. PCR assays are widely used in the detection and characterization of different foodborne pathogens like *S. aureus* (**Riyaz-Ul-Hassan** *et al.,* 2008), *L. monocytogenes* (**O'Grady** *et al.,* 2008), *Salmonella spp.* (**Choi** & **Lee**, 2004; **Malorny** *et al.,* 2007; **Murphy** *et al.,* 2007;
Perry *et al.,* 2007; **Stark** & **Made**, 2007), *E. coli* O157:H7 (**Velusamy** *et al.,* 2010), *Yersinia enterocolitica* (**Perry** *et al.,* 2007) and *C. jejuni* (**Ronner** & **Lindmark**, 2007).

During the amplification process, the DNA double strand separate into two single strands, each of which serves as a template for the production of another. This process involves three steps: the denaturation step in which the DNA is melted to convert the double-stranded DNA to single stranded DNA, the annealing step during which two primers are hybridized to the target positions in the DNA strands, and finally, the extension step in which the oligonucleotides are added to form the new strand catalysed by DNA polymerase.

1.5.2. Real-time PCR (qPCR)

Real-time PCR (qPCR or quantitative PCR) has ability to quantify bacterial pathogens by measuring gene numbers. It can be automated and is

relatively inexpensive and suitable for routine analysis (**Postollec** *et al.,* 2011). Quantitation depends on the measurement of the amount of DNA produced from each cycle of the amplification process, which is directly proportional to the amount of template DNA, which in turn, is directly proportional to the amount of analyte (microbes, viruses etc.). Consequently, real-time PCR can be used not only for detection of bacteria, but also to measure its amount in the sample (**Arya**, 2005). TaqMan primers and probes can be used in real-time PCR. The primers are labelled with fluorescent labels at the 5‘end. The probe is nonextendable at the 3‘end as not to work as a primer and has a fluorescent reporter dye attached to the 5‘end and a quencher dye attached to the 3‘end. When the probe is introduced into the PCR assay, it attaches to its complementary sequence on the DNA strand. During the exonuclease activity of Taq DNA polymerase enzyme, the primer is degraded and replaced by complementary nucleotides, so that the fluorescent and quencher groups are separated from each other. The quantity of fluorescent signal produced after every amplification cycle is directly proportional to the amount of DNA produced, which in turn is proportional to the amount of target DNA (**Gibson** *et al.,* 1996). The amplification process is illustrated in **Fig.** 1.18.

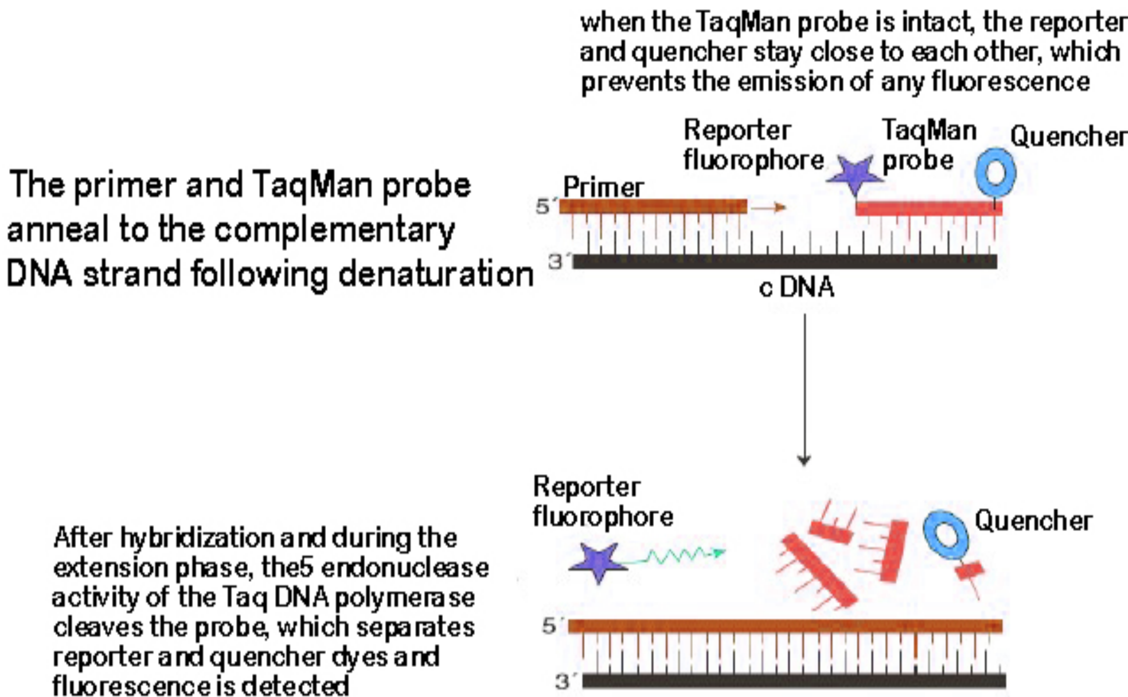


Fig. 1.18: Amplification process in real-time PCR: in the first step, the primer and TaqMan probe anneal to the complementary DNA strand following denaturation, hybridization and extension are completed in the second step (adapted from Arya *et al.,* 2005).

1.5.3. Nucleic acid sequence based amplification (NASBA)

Nucleic acid sequence based amplification (NASBA) (**Compton**, 1991) or transcription-mediated amplification (TMR) (**Gill** *et al.,* 2008) is characterized by isothermal processes and can be achieved at 41°C without using a thermocycler, therefore, it is considered to be more applicable and cheaper than other conventional PCR procedures. NASBA, as a molecular bacterial detection method, is a sensitive transcription-based system for the specific replication of nucleic acids (**Fakruddin** *et al.,* 2011). The final product is a RNA and cDNA mixture obtained from a

few RNA copies, which exponentially accumulates in the reaction medium, before finally being detected (**Fakruddin** *et al.,* 2011).

The amplification step in NASBA is usually achieved by the simultaneous action of three types of primers: (1) avian myeloblastosis virus reverse transcriptase (AMVRT), (2) T_7 RNA polymerase, and (3) RNase H in addition to two types of primers, P1 and P2, surrounding the sequences to be amplified (**Guatelli** *et al.,* 1990). The amplification process starts when primer P1 initiates the RNA reverse transcription (RT) reaction catalysed by a reverse transcriptase, then, the RNA strand in the RNA-DNA complex is degraded by the action of RNase H, the P2 starts the synthesis of a complementary DNA strand, then the T_7 RNA polymerase enzyme starts to divide the DNA double strand into two single strands preparing them for P1 to initiate a new copy of the RNA strand. This cyclic process is repeated indefinitely, producing a large number of RNA copies and cDNA double strands, with RNA single strands as the main product. (Ethidium bromide/agarose) gel electrophoresis or other fluorescent-based techniques can be used in detection step (**Guatelli** *et al.,* 1990). All reactions occur at a single temperature of 41°C, so the DNA of the target organism remains in the form of double strands and will not become a substrate for amplification (**Sargent** *et al.,* 2008).

1.5.4. Loop mediated isothermal amplification (LAMP)

Conventional and real-time PCR techniques are considered as sensitive and precise methods, but the nucleic acid amplification methods have their disadvantages such as high cost and risk of contamination in addition to the need for highly skilled workers. LAMP is a DNA

amplification based method that can overcome the disadvantages of both PCR and real-time PCR, such as high cost/time consumption and potential laboratory contamination, because it is simpler and has high efficiency amplification in addition to low cost (**Saharan** *et al.,* 2014). LAMP is a nucleic acid amplification test that uses a strand displacement polymerase to synthesize large amounts of DNA during a single temperature step of 60–65°C for less than 60 min (**Notomi** *et al.,* 2008). This technique has been developed by **Notomi** *et al.* (2000), who demonstrated it as a rapid, low cost, easy operated, highly sensitive, and specific detection method for application in different fields. In developing countries, LAMP assays are considered as a single temperature amplification and colorimetric detection method that is beneficial to reduce the system complexity of PCR-based methods and to solve the problems associated with traditional PCR, in addition to other advantages of nucleic acid amplification methods (**Notomi** *et al.,* 2000; **Soli** *et al.*, 2013). The most important advantages of these nucleic acid amplification techniques is that they do not require complex instrumentation and they provide comparable or even higher sensitivity and specificity to traditional PCR (**Zhao** *et al.,* 2014).

The sensitivity of the LAMP assay is less affected by the presence of inhibitory substances than PCR-based assays (**Kaneko** *et al.,* 2007). There are different methods used to detect the nucleic acid amplification in LAMP such as turbidity, fluorescence, intercalating dyes, gel electrophoresis, and pH indicators (**Goto** *et al.,* 2009). The LAMP reaction depends on an auto-cycling strand displacement DNA applied using isothermal conditions, with a set of four to six specially designed primers.

Furthermore, the specific amplification and detection of target DNA (6-8 specific regions of the target gene) can be completely performed in a single step without any advanced instruments (**Mori** & **Notomi**, 2009; **Zhao** *et al.,* 2014). The assay uses four primers, which are specifically designed to six target distinct regions of the DNA template; a further two loop primers can be used to accelerate the LAMP reaction (**Nagamine** *et al.,* 2002). LAMP products can be detected by the naked eye using SYBR Green I dye, instead of conventional gel electrophoresis analysis, once the colour of the solution changes to green in the presence of LAMP amplicons, while remaining orange for mixtures with no amplification (**Zhao** *et al.,* 2014). LAMP assays have been used for detecting pathogenic bacteria and viruses (**Itano** *et al.,* 2006; **Yamazaki** *et al.,* 2008; **Kurosaki** *et al.,* 2009) as well as yeast that cause food contamination (**Hayashi** *et al.,* 2007; **Wang** *et al.*, 2012; **Ishikawa** *et al.,* 2014). For instance, **Soli** *et al.* (2013) evaluated the sensitivity of LAMP colorimetric detection methods for *Salmonella, Shigella* and *Vibrio cholera*. However, the first foodborne pathogen application of the LAMP method was for the detection of stxA2 (toxin) in *E. coli* O157:H7 cells; higher-contrast pictures were obtained with this method than with traditional PCR (**Zhao** *et al.,* 2014). **Chen** *et al.* (2011) developed and evaluated a LAMP assay for the identification and direct detection of acidophilic thermophilic bacteria (ATB) in a juice sample. They demonstrated that this method could detect 2.25×10^{1} CFU/ml of ATB in 2 h.

1.5.4.1. Mechanism of LAMP

The amplification process in LAMP depends on six primers specifically designed to detect defined sites in the target DNA molecule (two outer, two inner and two loop primers). The two outer primers are referred to as the forward outer primer (F3) and backward outer (B3) and they have a role in strand displacement during the non-cyclic step. The two internal primers are the forward internal primer (FIB) and backward internal primer (BIP) and they help in the formation of two loops in both sides of DNA strand, where the amplification process proceeds at a constant temperature. The loop primers are known as the forward loop primer (FLP) and backward loop primer (BLP), these two primers are designed to accelerate the amplification process by binding to sites which cannot be accessed by the internal primers (**Parida** *et al.,* 2008). The amplification process in LAMP is achieved through two steps as shown in Figs. 1.19 and 1.20, the first is the non-cyclic step in which a DNA molecule with stemloops at each side is formed, this molecule serves as a starting sequence for the LAMP amplification in the next step, where the outer and inner primers are involved in the formation of starting sequence. In the next cyclic step, the loop primers, that are complementary to the loop ends of the starting structure, accelerate the amplification process. The LAMP amplification process can be achieved using only the outer and inner primers, that is without loop primers, but using loop primers decreases the time considerably (**Parida,** 2008).

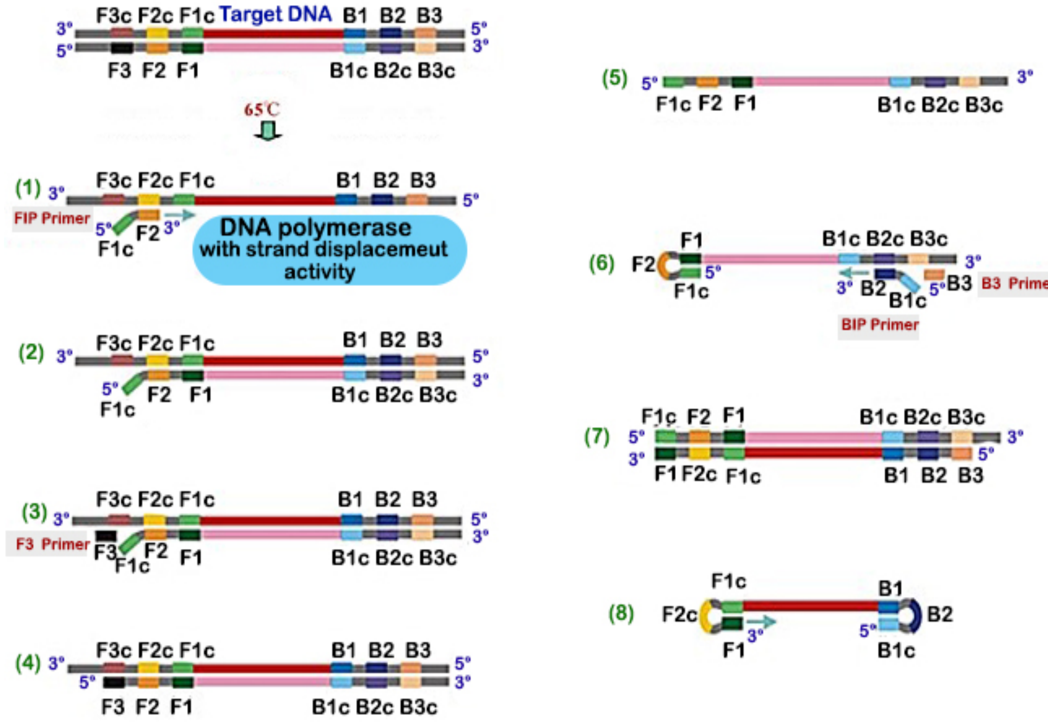


Fig. 1.19: Schematic diagram showing the non-cyclic step in the LAMP amplification process (from Parida, 2008).

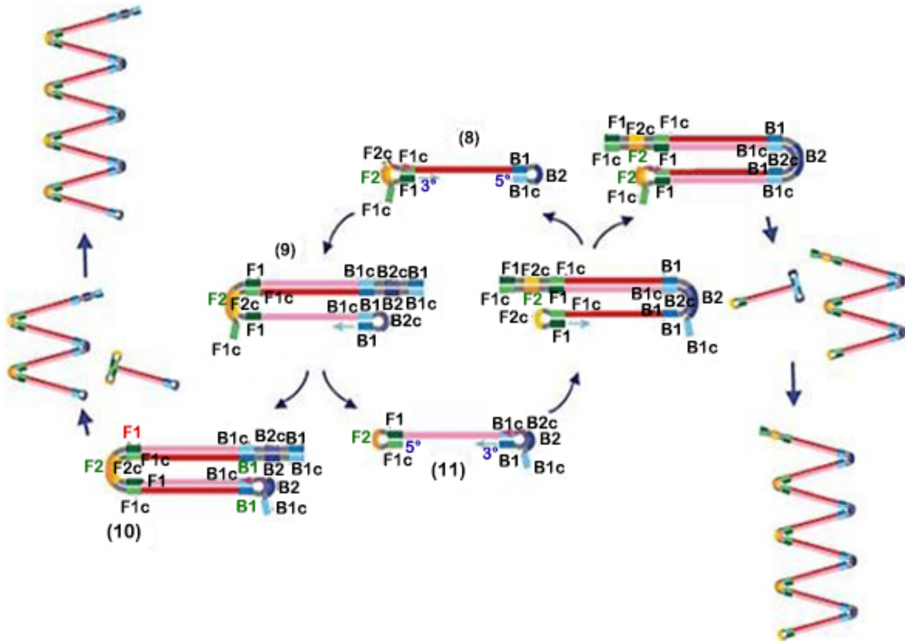


Fig. 1.20: The cyclic step in the LAMP amplification process (from Parida, 2008).

1.6. Nanotechnology

The application of nanotechnology in food safety requires collaboration between the fields of chemistry, biology, physics, engineering, and material sciences. Such collaborations can produce novel devices to improve food safety and quality. For example, biosensors can be used for the on-site analysis of food contaminates, such as detection of pathogenic and spoilage bacteria in addition to mycotoxins and other food contaminants like pesticides and other chemicals. The use of nanomaterials, structures such as semiconductors and conducting polymer nanowires, and nanoparticles (carbon nanotubes, silica nanoparticles, dendrimers, noble metal nanoparticles, gold nanoshells, superparamagnetic nanoparticle quantum dots, polymeric nanoparticles) is increasing rapidly (**Katz** *et al.* 2005). Furthermore, the use of these devices is cost effective and time saving in comparison with other conventional detection methods. Nanotechnology has been applied in biosensor engineering to fabricate transducer devices and in the recognition of ligands and labels. Indeed, the use of nanomaterials in the manufacturing of biosensors yields many advantages, such as high sensitivity because of the larger available surface area and by using nanoparticles as a label, miniaturization is also possible (**Jain**, 2005).

1.6.1. Nanospheres-based bioassay

Nanospheres-based bioassays are rapid detection methods in which nanospheres provide a solid phase substrate for bio-affinity binding similar to the walls of traditional test tubes and microtiter plates (**Soini** *et al.,* 2004). These nanospheres are coated with biological molecules,

such as antibody where the antigen-antibody reaction occurs on its surface. The commercially available nanospheres can be manufactured in different sizes from many materials, such as polystyrene, acrylate, and glass. They can be activated by functional groups to provide chemical binding sites and also impregnated with fluorochromes and magnetic groups for identification or isolation purposes. The use of nanospheres in a bioassay provides a rapid single step reaction in which all reagents and sample are mixed in the same site. Many articles have reported using nanomaterials in the development of biosensors for bacterial detection, as shown below in Table 1.1.

Table 1.1. List of nanomaterials used for the detection of bacteria

Reference	Type of nanoparticle	Bacteria detected	Method
Joo *et al.*, 2012	Superparamagnetic Fe_3O_4 nanoparticles functionalized with monoclonal antibodies toward *Salmonella*	*Salmonella*	Immunomagnetic separation
Wang *et al.*, 2016	Polyethyleneimine (PEI)-modified Aucoated magnetic microspheres (Fe_3O_4@Au@PEI) and concentrated Au@Ag nanoparticles (NPs),	*E. coli* , *S. aureus*	SERS detection method
Qi *et al.*, 2016	Cadmium sulphide (CdS) nanoparticles	*Desulforibrio caledoiensis*	Fluorescence microscopy
Gao *et al.*, 2006	FePt@Van magnetic nanoparticles,	*E. coli Coagulase-negative Staphylococcus (CNS)*	Fluorescence microscopy
Raj *et al.*, 2015	Cysteine gold nanoparticles (CAuNPs)	*E. coli*	Colourimetric method
Li *et al.*, 2013	Streptavidin coated magnetic nanoparticles	*E. coli, Salmonella, Vibrio cholera, C. jejuni*	Multiplex PCR

Cao *et al.*, 2011	Bimetallic Au@Ag core–shell structures	*C. jejuni*	Immunomagnetic separation-polymerase chain reaction (IMS-PCR) method
Sepunaru *et al.*, 2015	Silver NPs (AgNPs)	*E. coli*	Anodic particle coulometry technique
Reference	**Type of nanoparticle**	**Bacteria detected**	**Method**
Wang *et al.*, 2016	Au-coated magnetic nanoparticles (AuMNPs) conjugated with *Staphylococcus aureus (S. aureus)* antibody	*S. aureus*	SERS detection method
Cao *et al.*, 2014	Molecular beacon–Au nanoparticle	*E. coli*	Real-time PCR
Zhang *et al.*, 2012	Multifunctional magnetic–plasmonic Fe_3O_4–Au core–shell nanoparticles (Au-MNPs)	*E. coli P. aeruginosa A. calcoaceticus*	SERS detection method
Wang *et al.*, 2014	CdSe/ZnS@SiO_2–NH_2 nanoparticles	*Salmonella typhimurium, E. coli S. aureus*	Fluorescence microscopy
Zhou *et al.*, 2014	Silver nanoparticles	*E. coli ,Staphylococcus epidermidis*	Dynamic SERS
Wu *et al.*,2014	Multicolour upconversion nanoparticles coupled with magnetic nanoparticles	*S. aureus , Vibrio paraheamolyticus, and S. typhimurium*	Multiplexed Luminescence Bioassay Method

1.6.2. Optical flow cytometry assay

This type of the assay was designed for the characterization of different cell types, based on specific fluorochrome labelling of cell antigens. The use of flow cytometry and nanospheres with specific biological binding activity for the detection of analytes was performed in different sample

liquids (**Ashcroft** *et al.,* 1988). Recently, a number of flow cytometry-based detection methods have been developed for bacterial detection. **Xue** *et al.* (2016**)** described a fluorescence-labelled oligonucleotide probe specifically binding a 16S rRNA for the detection of *E. coli.* **Ahmed** *et al.* (2016**)** reported a flow cytometry-based immunomagnetic separation (IMS) method for the isolation and enrichment of *S. entirica serovar typhimurium* from liquid samples**.**

1.7. Biosensor techniques

1.7.1. Introduction

The International Union of Pure and Applied Chemistry (IUPAC) defined a biosensor as a self-contained integrated device, which is capable of providing specific quantitative or a semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with a transduction element (**Thevenot** *et al.,* 1999). Otherwise, biosensors are defined as a bio-analytical device incorporating a molecular recognition element associated or integrated with a physicochemical transducer (**Tothill** & **Turner**, 2003). Biosensors use a combination of biological receptors, which may be antibodies, enzymes, nucleic acids etc, and a physical or physiochemical transducer (**Leonard** *et al.,* 2003). The receptor plays a role in the recognition of analytes and the transducer transforms the reaction on the bioreceptor, such as an antigen-antibody interaction, into a measurable response or signal which can be measured by the detector. **Fig.** 1.21 shows the main components of biosensor.

The antibody–antigen interaction induces a physico-chemical change at the bioreceptor or biointerfaces, e.g. change of mass, heat change or change in electrical potential, which is converted by the transducer to an electrical signal. The output from the transducer is then amplified, processed and finally displayed as a measurable signal. Such a combination of bioreceptor and transducer enables the measurement of the target analyte without the use of reagents (**Davis** *et al.,* 1995).

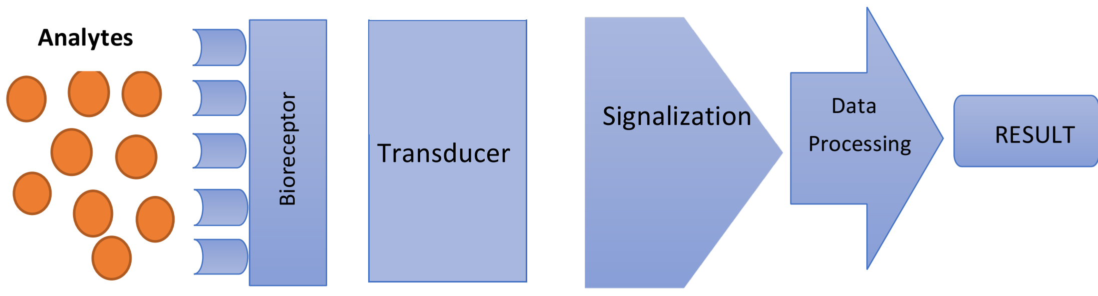


Fig. 1.21: Basic components of a biosensor.

1.7.2. Classification of biosensors

As mentioned above, biosensors are composed of two main components which are bioreceptors and transducers, each of which are classified into several types.

Biosensors can also be classified according to the type of bioreceptor or transducer as illustrated in **Fig.** 1.22.

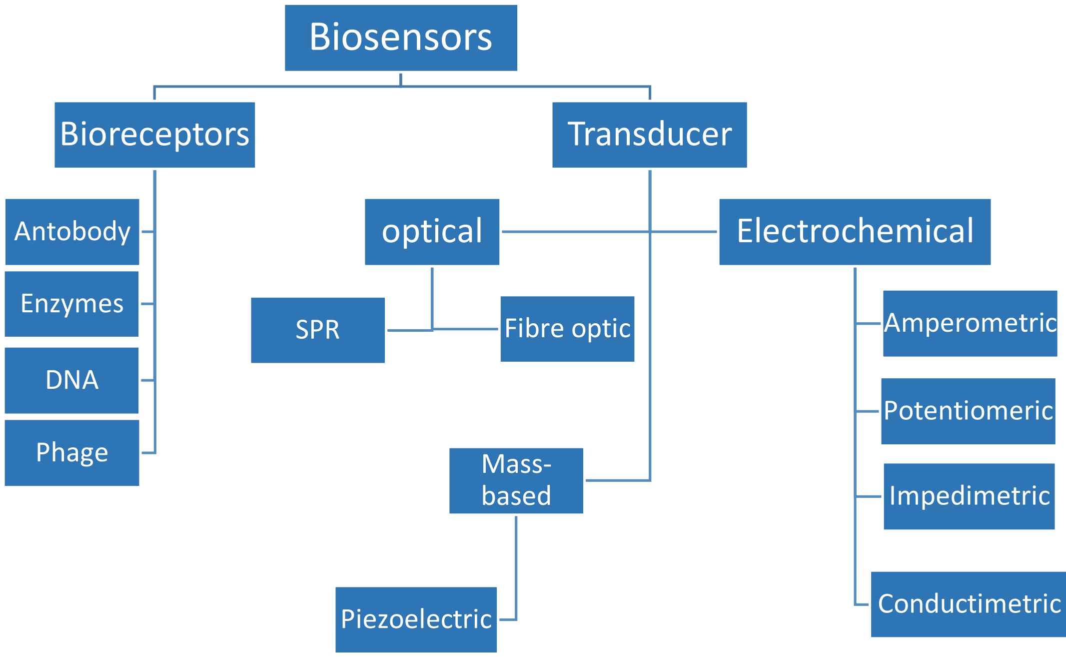


Fig. 1.22: Classification of biosensors (Velusamy *et al.,* 2010).

1.7.2.1. Bioreceptor

Bioreceptors are biological species which are responsible for the capture of the analyte on the sensor surface. They are usually immobilized into a suitable substrate of the biorecognition element of the biosensor. The efficiency and specificity of the biosensor is mainly related to these bioreceptors which bind specifically to the analyte of interest. There are a number of bioreceptors used in biosensors as mentioned below.

1.7.2.1.1. Antibody bioreceptors

Antibodies are important groups of biological species commonly used in immune sensors such as bioreceptors, due to their highly specific way of binding to their antigens, described by **Vo-Dinh** and **Cullum** (2000) as a key and lock fit. Antibodies are the preferred bioreceptor due to the three-dimensional way in which they bind to their antigens, which

provides the ability for antibodies able to bind a large variety of antigens with different molecular shapes (**Velusamy** *et al.,* 2010). These advantages of antibody as a bioreceptor make immune sensors efficient analytical tools for the detection of a variety of analytes, including biomolecules, chemicals and microorganisms (**Baccar** *et al.,* 2010; **Braiek** *et al.,* 2012; **Ahmed** *et al.,* 2013; **Piro** *et al.*, 2016; **Christopher** *et al.,* 2017).

1.7.2.1.2. Enzyme bioreceptors

Most enzymes are proteins, except for a few ribonucleoprotein enzymes. These enzymes can be used as bioreceptors due to their specific binding capability and also their reactivity which improves the sensitivity of the sensor. In most cases, enzymes are used as a label rather than a bioreceptor in immunological methods. The enzyme-labelling process of antigens or antibodies replaces other labelling methods, such as radioisotopes and fluorescent tag, due to the higher sensitivity and the stability of enzymes. Enzymes can be also used as a biorecognition element in biosensors for the detection of bacteria in food samples (**Bulbul** *et al.,* 2015; **Wu** *et al.,* 2016).

1.7.2.1.3. Nucleic acid and aptamer bioreceptors

The use of DNA as a bioreceptor is based on the fact that each organism has its own unique DNA sequence and the identification process depends on matching between complementary base pairs. Biosensors utilizing DNA as a bioreceptor are simpler and inexpensive, in addition to being rapid. **Sergeev** *et al.* (2004) used DNA microarrays for the simultaneous

detection of *Listeria spp., Campylobacter spp., S. aureus* enterotoxin and *clostridium perfrinigens* enterotoxin. In another study, a single stranded aptamer was used in a rapid method for the quantitative detection of food pathogens (**Kim**, 2007). Many applications have used aptamer-based biosensors for the detection of mycotoxins (**Rhouati** *et al.,* 2016)**.** It has also been reported that an electrochemical aptasensor was used for the detection of *Salmonella* (**Li** *et al.,* 2016**).**

1.7.2.1.4. Bacteriophage-based bioreceptor

Bacteriophages (phages) are viruses which bind specific receptors on bacterial cell surfaces in order to inject their genetic material inside the cell. These phages recognize bacterial receptors by using its tail spike proteins. This ability of the phages to specifically recognize bacterial cells makes them a good biological receptor, which have been used in the detection of many pathogenic bacteria like *E. coli* (**Singh** *et al.,* 2009), *S. aureus* (**Balasubramanian** *et al.,* 2007), *B. anthracis* spores (**Huang** *et al.,* 2008; **Xie** *et al.,* 2009), *Salmonella* (**Wang** *et al.,* 2017) and *E. coli* & *Salmonella* (**Vinay** *et al.,* 2015).

1.7.2.2. Transducers

The transducer is one of the two main components which plays the main role in producing a measurable signal related to the concentration of analyte and as mentioned before, biosensors can be classified into different types based on the type of transducer.

1.7.2.2.1. Optical-based biosensors

Optical biosensors measure the change in characteristics of the sensor surface caused by the reaction between the analyte and the sensing layer (**Narsaiah** *et al.,* 2012). These characteristic changes mainly include changes in amplitude, frequency and polarization of light. Optical-based biosensors can be classified into subclasses according to the type of reaction between light and the complex by the binding between the analyte and bioreceptor. Different types of optical biosensors for the detection of bacteria in different matrices have been reported (**Yoo** *et al.*, 2010; **Luo** *et al.,* 2013; **Tissari** *et al.,* 2014; **Ahmed** *et al.,* 2014; **Pires**, *et al.,* 2014; **Tokel** *et al.,* 2014).

1.7.2.2.1.1. Surface plasmon resonance biosensors

Surface plasmon resonance (SPR) is the type of optical biosensors which utilizes reflectance spectroscopy. The device measures changes in the refractive index of the sample medium due to the binding between the analyte and bioreceptor, which is fixed on the sensor transducer. It can also measure the change in the angle of reflected light related to the change in the density of the sample solution, which can give an indication of the analyte concentration. Many pathogenic bacteria have been detected in foods by using this type of biosensor, such as *Salmonella spp.* *(***Bhunia** *et al.*, 2004), *E. coli* 0157 (**Meeusen** *et al.,* 2005), *L. monocytogenes* (**Taylor** *et al.,* 2006), *C. jejuni* (**Masdor** *et al.,* 2017**)** and *E. coli* **(Yamasaki** *et al.,* 2016).

1.7.2.2.1.2. Optical fibre-based biosensors

In optical fibre-based biosensors, the total internal reflection (TIR) principle is utilized to transfer the light beam to and from the sample solution through either an optical fibre or wave guide. What makes this type of sensor excellent for detection is the sensitivity of the light propagating through fibres or wave guide to any minor changes in the surrounding, which results in the high sensitivity required for pathogenic detection in food and other applications (**Velusamy** *et al.,* 2010). Optical fibre-based biosensors are basically composed of a light source, transmission medium (fibre or wave guide), immobilized biological element (enzymes, antibodies, microbes), optical probe and optical detection system (**Narsaiah** *et al.,* 2012). Previous studies have used this type of biosensor for the detection of pathogenic bacteria in foods such as *L. monocytogenes* (**Strachan** & **Gray**, 1995*)*, *Salmonella spp.* (**Morgan** *et al.,* 2006), and *E. coli 0157* (**DeMarco** & **Lim**, 2002) and also for the detection of preservatives in milk (**Saracoglu** *et al.*, 2016**).**

1.7.2.2.2. Electrochemical biosensors

Electrochemical biosensors depend on the chemical reactions occurring in the sample medium producing or consuming ions or electrons, leading to a change in the electrical characteristics of the medium, which can be evaluated to measure the concentration of analyte. Electrochemical biosensors are classified according to the observed parameters into potentiometric, amperometric, impedimetric and conductometric biosensors (**Velusamy** *et al.,* 2010). Many recent articles have reported the use of electrochemical biosensors for the detection of pathogens. **Li**

et al. (2016) developed an electrochemical aptasensor for the rapid and sensitive determination of *Salmonella.* **Zhou** *et al.* (2015) used a phase-based electrochemical biosensor for the detection of *E. coli* in food samples.

1.7.2.2.3. Piezoelectric (mass sensitive) biosensors

The principle of this type of biosensor depends on the coupling between a material with piezoelectric properties, such as quartz, oriented zinc oxide and aluminium nitride, and biological elements, where binding between biological molecules, like antigen and antibody, on the surface of such piezoelectric materials leads to a change in the oscillation frequency of the crystal, which can be measured as an electric charge (**Monošík** *et al.,* 2012). The piezoelectric transducer provides the label-free detection of biological molecules like bacteria and can be used for many analytical purposes in environmental fields and food safety purposes.

1.7.2.3. Summary of using different transducers in biosensors

As mentioned before, biosensors can be classified into different groups based on the types of biorecognition elements and transducers. Each one of these types has some advantages and disadvantages in terms of selectivity, sensitivity, cost, time consuming and simplicity / complexity. Regarding to electrochemical and piezoelectric biosensors, using all of these techniques makes the analytical process faster but also complex and more costly. By contrast, using optical biosensors makes the analytical process more easy and more sheep so it is more suitable for

food analysis which need fast and easy process. So, in our work we concentrated on developing detection kits based in this type of transducers.

1.7.2.4. Comparison between the different methods of pathogenic detection

As mentioned before, there are many methods used for pathogenic detection, each one of these methods rely on a specific technique, and each of these method has its advantages and disadvantages. In the following **table (1.2)**, a comparison between these methods and techniques in term of accuracy, sensitivity, time consuming, cost, repeatability and reliability.

Table 1.2: comparison between pathogenic detection methods

Technique	Method	Advantages	Disadvantages
Traditional	Culture method	1. The most reliable and accurate techniques for the detection of foodborne diseases.	1- Time consuming 2- cultivation runs the risk of contamination with commensal flora and the possibility of reduced viability during transportation
	Microscopy	1- Simple technique and easy to use	1- limited sensitivity

Immunoassay	ELIZA	1- Accurate and sensitive method for detecting antigens or haptens 2- The detection of an analyte can be achieved in both liquid reagent and by dry strips	1- Sensitivities of commercial ELISAs are widely different depending on sampling times and processing methods. 2- The possibility of giving false negative results.
	Lateral flow immunoassay	1- Simple device easy to use and interpret results. 2- Does not require any washing or a manipulation.	1- Low sensitivity and reproducibility. 2- Has limitations in quantitative analysis.
	Paper-based microfluidic immunoassay	1- Small sample size. 2- Short duration of analysis. 3- Good sample uniformity 4- Colorimetric.	1- Some modes of wax patterning suffer from inflexibility and low reproducibility between batches.
	Immunomagnetic separation assay	1- Well-established tool for the specific separation of bacterial contaminations from complex matrices. 2- Many detection methods can be used, involving cultivation methods and molecular methods or immunoassay methods	Mainly, used for separation other than being a detection technique.

Molecular	PCR	1- Accurate and reliable. 2- Less time-consuming than traditional methods. 3- Doesn't need pre-enrichment step. 4- Possibility of detecting more than one pathogen simultaneously.	1- Unable to distinguish between viable and inviable cells. 2- Costly 3- Need highly skilled technicians.
	Real-time PCR (qPCR)	1- Accurate and reliable. 2- Less time-consuming than traditional methods. 3- Doesn't need pre-enrichment step. 4- Possibility of detecting more than one pathogen simultaneously. 5- Quantitative analysis.	1- Unable to distinguish between viable and inviable cells. 2- Costly 3- Need highly skilled technicians. 4- Amplification can be inhibited by components in sample matrix.
	NASBA	1- Isothermal so, no need for costly thermocycler. 2- Sensitive 3- Suitable for RNA amplification.	1- Low repeatability and reproducibility comparing with PCR.
	LAMP	1- Isothermal amplification for DNA molecules. 2- Low cost 3- More efficient that PCR 4- Possibility of colorimetric detection. 5- Easy to perform analysis without the need to highly skilled technicians.	1- Less availability of reagents. 2- Complexity of primer design. 3- This technology, does not allow the inclusion of an internal PCR inhibition control (IC) and for this reason it is sometimes necessary to perform the reactions in duplicate, one reaction for the target and the other one for the IC.

Biosensors	Biosensors.	1- Rapid and contentious measurement. 2- High specificity. 3- Very less usage of reagents required for calibration 4- Fast response time 5- Suitable for applying in various applications like environmental , food safety , medical …etc.	➢ Disadvantages of each type of biosensors are related to the type of bioreceptor and transducer used in that biosensor.

2. Aims and Objectives

2.1. Aims

The aims of this project were to develop a number of suitable on-site rapid screening kits for foodborne diseases including *Salmonella enteritidis, Salmonella typhimurium, Campylobacter jejuni* and *Staphylococcus aureus*. Then, develop a confirmation test for each one using a biosensor and nanoparticles.

2.2. Objectives

To achieve the aim of the project, the objectives were as follows:

1. Development of a low cost nano-based immunosensor for the detection of pathogenic bacteria in poultry processing plants.

2. Development of a general bioreceptor (lactoferrin) nano-based immunosensor for the detection of pathogenic bacteria.

3. Development of a new fast and simple colorimetric technique for detecting the loop mediated isothermal amplification (LAMP) product of pathogenic bacteria in food samples.

4. Development of a labelled aptamer-based immunosensor for detecting pathogenic bacteria in food samples.

5. Development of a label-free immunosensor for detecting *Norovirus* in water and food samples.

6. Development of confirmatory test for each method.

2.3. Study hypothesis

Traditional methods for detecting bacteria are no longer able to meet the analytical needs in many areas, such as quality assurance systems, so there is a need to develop new quick and easy methods to detect bacteria to meet such requirements.

Chapter 2

Experimental procedures

2.1. Materials

*Salmonella typhimurium (*St) (ATCC 14028), *Salmonellae enteritidis (Se)* (ATCC 13076), *Staphylococcus aureus (*Sa) (ATCC 6538), *Listeria monocytogenes* (Lm) (ATCC 7644), E. *coli* (Ec) (ATCC 8739) and *Campylobacter jejuni* (Cj) (ATCC 29428) were purchased from the American Type Culture Collection (Manassas, VA). Stock cultures of all strains, except Cj, were stored at -80°C in 20% glycerol solution. Prior to use, the frozen culture was activated in tryptic soy broth (TSB, Oxoid LTD, Hampshire, UK) at 37°C, with two consecutive transfers after 18 ± 2 h incubation periods. The culture was centrifuged at 10000 X g for 10 min at 4°C and washed twice with TSB. Cell suspensions were prepared and the optical density (OD) at 600 nm adjusted to 0.5, which is equivalent to 10^{8} CFU/ml. Then, serial dilutions from 1 to 10^{8} CFU/ml were prepared in TSB.

Stock cultures of Cj were grown for 4 h at 37°C, then for 24-48 h at 42°C under microaerophilic conditions in Bolton broth media (Oxoid LTD) in an anaerobic jar, with an active catalyst and a microaerophilic gas generator pack. Serial 10fold dilutions were made in Maximum Recovery Diluent (Oxoid LTD) and the viable cell numbers of *Cj* were determined by surface plating on Columbia Blood agars (Oxoid LTD). Blue, orange and green dye coated nanobeads (50 nm bead size) with carboxylic acid functional groups were obtained from Bangs Laboratories Inc. Carboxylic acid cobalt based magnetic nanoparticles with a diameter of 50 nm were purchased from Turbobeads (Zurich, Switzerland).

Monoclonal S. *enterica serovar typhimurium*, S. *enterica serovar entritidis* (specific for lipopolysaccharides) , Lm (specific for whole cell) , Ec (specific for somatic antigen) and Cj (specific for the non-flagellar antigen), all are with concentration of 4mg/ml. Sa polyclonal antibody were purchased as purified liquid from Biospacific (Emeryville, CA, USA). Lactoferin was from Monojo (Amman, Jordan) and Loopamp^{TM} detections kits for *Salmonella*, *Campylobacter* and E. *coli* O157 were from Eiken Chemical Co. Ltd (Japan). $NaIO_4$, 10x PBS buffer, bovine serum albumin (BSA), 1-ethyl-(3 dimethylaminopropy) carbodimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. Colloidal gold nanoparticles (30 nm) and anti*Norovirus* specific antibodies GI Mab (cloneNG28) and anti-*Norovirus* GII Mab (clone NP8) were purchased from Atlas Medical Company (Aman, Jordan). Reagents for DNA extraction were from QIAGEN (UK). HPLC grade chloroform was obtained from Scharlau (Spain).

2.2. Methods

2.2.1. Activation of cellulose (cotton swab)

Sterilized cotton swaps were activated by immersing in a mixture of 100 ml of 2M sodium periodate ($NaIO_4$) and 0.18M sulphuric acid overnight at room temperature as shown in **Fig.** 2.1.

Cellulose + $NaIO_4$ → Dialdehyde cellulose + $NaIO_3$ + H_2O

Fig. 2.1: Schematic diagram explaining the production of dialdehyde cellulose (DIC) from cellulose through periodate oxidation of cellulose.

2.2.2. Immobilization of protein (antibody/lactoferrin) on cotton swabs for immunoassay

The cotton swabs were washed with cold distilled water to remove excess of oxidizing agents and ready for further use without drying.

The activated aldehyde group in the cotton swab was used to couple the amine group present in the antibody. Briefly, 40 µl of antibody for the targeted bacteria (1 mg/ml) was mixed with 1 ml of PBS (pH 7.4) for 3 minutes. Then, the activated cotton was immersed in the antibody buffer solution overnight at 4°C. The antibody linked cotton swab was washed with PBS buffer to remove the unbound antibodies. The excess aldehyde groups were blocked with BSA by incubating the cotton in 1 ml of BSA (1 mg/ml) for 30 minutes at room temperature, followed by 3x washes with PBS. The antibody linked cotton was stored at 4°C in PBS for further use. The control samples were prepared by immersing the activated cotton swabs in 1 mg/ml solution of BSA in PBS overnight at room temperature.

The BSA linked cotton swab was extensively washed with PBS to remove the unbound BSA. BSA linked control samples were stored in PBS buffer.

2.2.3. Immobilization of antibody on the beads for immunoassay

The immobilization of antibody on the beads for immunoassay, except gold nanoparticles, was performed according to Sohn and Lee (2014). Briefly, 300 µl of each (blue, orange and green) beads or magnetic bead suspensions were washed 3 times using 300 µl of water. The 1-Ethyl-3-(3-dimethylaminopropyl) carbodimide/N-hydroxysuccinimide (EDC/NHS) solution was prepared by mixing 100 mg of 1-ethyl-(3-dimethylaminopropy) carbodimide hydrochloride (EDC) and 100 mg of NHS in 10 ml of water. Then, 300 µl of EDC/NHS solution was added to the beads and mixes for 20 min at room temperature. The EDC/NHS activated beads were washed 3x using PBS buffer, followed by the addition of 20 µl of antibody and 300 µl of PBS. A specific antibody for each bacterial strain was mixed with a different bead. For example, Se specific antibody was incubated with blue nanobeads, St specific antibody was incubated with black magnetic nanobeads, Sa specific antibody was incubated with orange nanobeads, and Cj specific antibody was incubated with green nanobeads overnight at 4°C. Antibody linked nanobeads were washed with PBS to remove excess antibody. Finally, the active sites were blocked by mixing the nanobeads with 1 mg/ml of BSA in PBS for 30 min. Unbound BSA was washed with PBS and the antibody bound nanobeads were stored at 4°C in PBS buffer. For gold nanoparticles, immobilization of secondary anti-*Norovirus* antibody was

carried out physically by incubation of the antibody with the solution of gold nanoparticles for 1 hour.

2.2.4. Immunoassay screening procedure

The screening procedure involved two steps as shown in (fig. 2.2) The specific capture primary antibodies (C-mAb) were conjugated to the cotton swap and the developing colour solution consisted of a cocktail of magnetic beads or coloured polymeric nanobeads conjugated to secondary detection antibody (DmAb). In the first step, the cotton immobilized specific antibody was swabbed over surfaces to pre-concentrate the bacteria from artificially contaminated surfaces with a serial dilution of target bacterial cells. *S. typhimurium, S. enteritidis, S. aureus and C. jejuni* were used for artificially contaminating the surfaces of the chicken, glass slide and stainless-steel surfaces. The number of bacteria on each surface was confirmed by swabbing and culturing the cells, followed by colony counting. The cotton immobilized antibody-bacteria complexes were washed with PBS buffer twice to remove free cells from the cotton.

The second step is the detection step, in which a bacterium was sandwiched between the cotton immobilized antibody and secondary detection antibody conjugated to coloured nanobeads. The cotton swap antibody-bacteria (cottonmAb-cell) complexes were immersed in the developing colour solution in PBS for 2 min. The cotton-primary antibody-cell-secondary antibody (cotton-C-mAbcell-mAb-D) sandwich complex was washed with PBS buffer to remove the unbound beads. The colour of the beads bound to the cotton swap indicated the specific

bacterial stain present on the contaminated surfaces as shown in **Fig.** 2.1. Control samples were prepared as above, except the surfaces of the chicken, glass and stainless-steel were not contaminated with bacteria (see **Figs**. 2.2 and 2.3).

In the specificity assay, the specific binding of each bacterium was tested by incubating the specific primary antibody immobilized cotton swapped over surfaces contaminated with different bacteria and washed with PBS. Then, cotton swaps were immersed in four different secondary antibodies conjugated to coloured nanobeads for 2 min. Finally, the cotton swaps were washed with PBS to remove the unbound beads. In the case of magnetic beads, the unbound particles were separated by passing the cotton swab over a small magnetic sheet. When the colour of the cotton surface matched the colour of the secondary antibody conjugated to nanobeads, this indicated the specific binding of the target of interest.

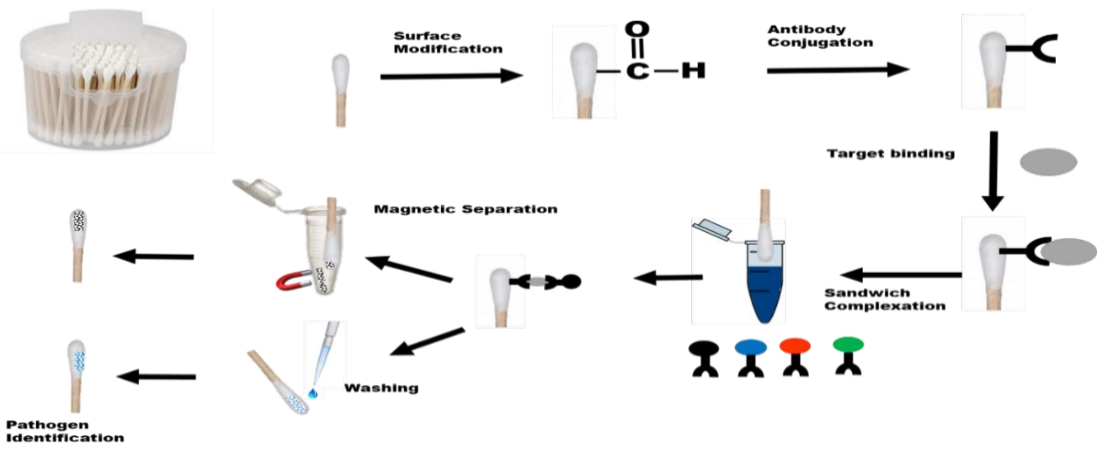


Fig. 2.2: Schematic diagram for the antibody immunoassay procedure.

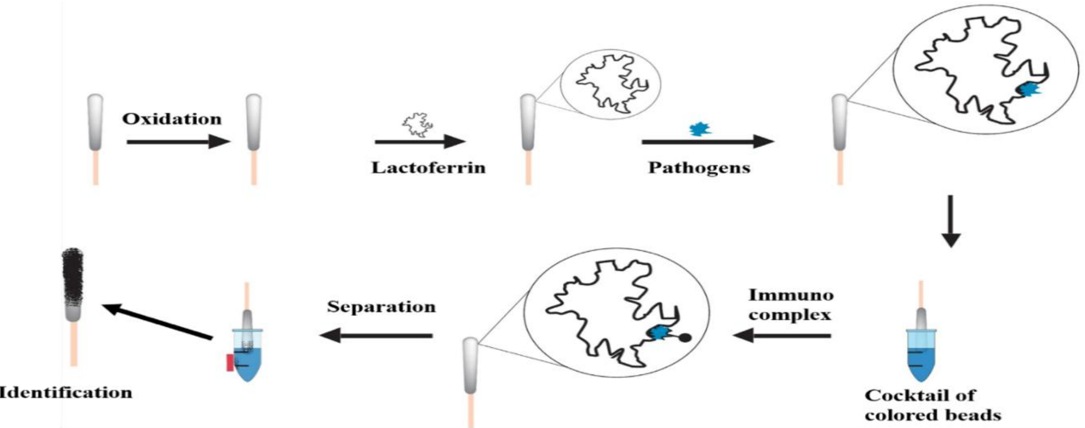


Fig. 2.3: Schematic diagram for lactoferrin immunoassay procedure.

2.2.5. Sample pre-treatment for LAMP amplification (DNA extraction)

Contaminated stainless-steel surfaces were swabbed using cotton buds with different concentrations of pre-enriched cultures (10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10 CFU/ml). The alkali heat extraction for DNA was performed by dipping the contaminated cotton into a tube containing 50 µl of extraction solution (EXF), the mixture was incubated at 95°C for 5 min. The solution was cooled on ice, 10 µl of 1M Tris-HCl was added, mixed well with a vortex mixer, followed by centrifugation at room temperature at 14000 xg for 1 min. The resulting supernatant was then used in the next amplification step.

2.2.6. Preparation of master mix for LAMP procedure

The required volume of master mix was prepared by mixing the following for each sample: 12.5 µl of 2x reaction mix (RM), 2.5 µl (16 µM FIP, 16 µM BIP, 2 µM F3, 2 µM BE, 4 µM LoopF, 4 µM LoopB) of primer mix (PM 0157), 4 µl of distilled water, and 1 µl of Bst polymerase, to a total volume of 20 µl. The solution was mixed well by gently tapping the tube, inverting the tube, or 3 x 1 sec pulses by vortex mixer. The solution was collected at the bottom of the tube by centrifugation and used as soon as possible.

2.2.7. Amplification step in LAMP

LAMP amplification was performed by mixing 20 µl of master mix containing (primer mix, dNTPs, buffer solution and template DNA) with 5 µl of sample solution, positive or negative control (EXF solution) in the reaction tube, before incubation at 65°C for 40 to 60 min in a simple heating block. The amplification product was detected by the addition of 5 µl of lactoferrin immobilized nanobeads (coated polymer nanobeads solution), followed by gentle shaking in order to check aggregation of dye beads by DNA amplification as explained in **Fig.** 2.4. details of target genes and primer sequences are provided in table 2.1.

Table 2.1 sequences of LAMP primers and target genes.

Microorganism	Primers sequences	Gene	Reference
Campylobacter jejuni	FIP CTGCTGAAGAGGGTTTGGGTGCATATTGTGCCATCCAA BIP GCTAAATACTTTGCAGCAAGCAGCTTTGCCTTTACAAGAA TGC LF GGTGCTAAGGCAATGATAGAAG LB CATCATGACCGCAAGCATG *F3* GAAGAAG C C AT C AT C GC A *B3* AATAGGACTTCGTGCAGATATG	*hip0*	(HEE-JIN DONG et al. 2014)
Escherichia coli 0157:H7	FIP GCTCTTGATGCATCTCTGGTACACTCACTGGTTTCATCAT ATCTGG BIP CTGTCACAGCAGAAGCCTTACGGACGAAATTCTCCCTGT ATCTGCC F3 CAGTTATACCACTCTGCAACGTG B3 CTGATTCGCCGCCAGTTC Loop F1 TGTATTACCACTGAACTCCATTAACG Loop F2 GGCATTTCCACTAAACTCCATTAACG	*VT2*	(Yukiko HaraKudo et al. 2008)

Salmonella	Sa-FIP GGCGTGAGAGATCCACCTGGAATGCGCCGTAATAGCGGTC Sa-BIP CACCATTATGGAAACGCTTATCCGCCGGATACAGCTGAAGCATC Sa-LF CAGGTGATCAACATCCCGCC Sa-LB CGGTAAAGTGGTCAGCAAAGAT Sa-F3 GCCATTCCACATCGAAGAGGT Sa-B3 ATGAGAACATCAATGGTATGGC	*phoP*	(Kevin W. Soli et al. 2013)

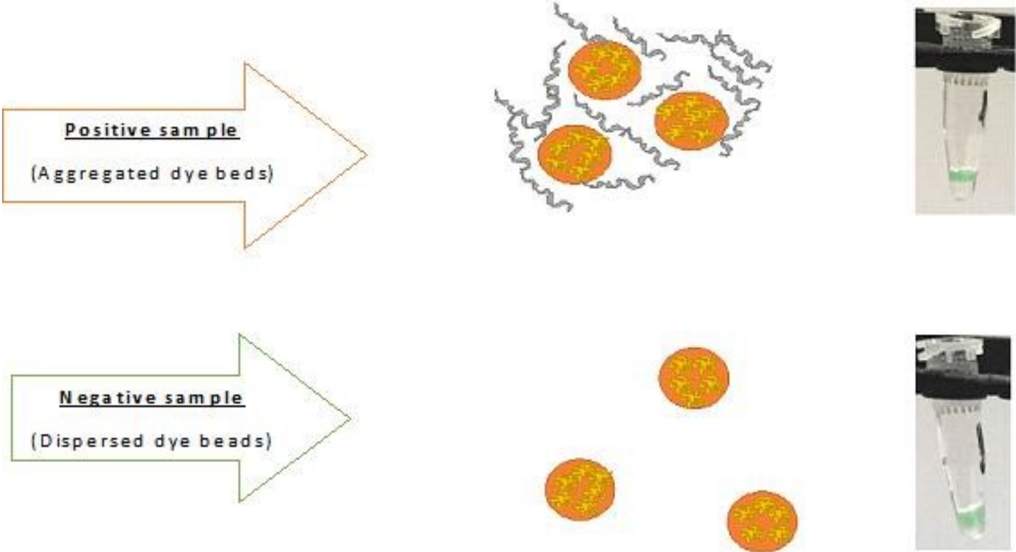


***Fig.* 2.4: Schematic diagram for LAMP procedure**

2.2.8. DNA extraction for real-time PCR

The cotton swabs with bacteria captured either by antibodies or lactoferrin were placed in 2 ml centrifuge tubes and 1 ml of lysis buffer containing (cetyltrimethylammonium bromide) and 2.5 µl of proteinase were added, before briefly vertexing the mixture. The solution prepared in the previous section was incubated in a water bath at 60°C for 30 min (under continuous shaking), then cooled to room temp on ice to enhance precipitation of the inhibitors, before centrifugation for 5 min at 2500 x g. Then, 700 µl of the mixture was aliquoted into a 2 ml centrifuge tube,

500 µl of chloroform was added and vortexed for 15 s, prior to centrifugation for 15 minutes at 14000 x g. The supernatant (350 µl) was transferred to a new tube and 350 µl of PB buffer containing guanidine hydrochloride and isopropanol was added and mixed well by vortex. The solution was transferred to the QIAquick spin column placed in a 2-ml tube, then centrifuged at 17.900 x g for 1 min. The flow-through was discarded and the collection tube was used again for washing by adding 500 µl of the AW2 buffer, centrifuged for 1 min at 17.900 x g and for dried by centrifugation at 17.900 x g for 1 min. For elution, the spin column was transferred to a new tube, 150 µl of elution buffer (EB buffer) was added to the column, then incubated for 1 min at room temperature and centrifuged at 17.900 x g for 1 min.

2.2.9. Real-time PCR analysis

Preparation of master mix

According to the number of reactions, the required volume was prepared plus an additional 10% volume to compensate for reagent loss according to the table below in table 2.1.

Table 2.1 Example for calculation and preparation of 10 reactions

Components of master mix	Amount per reaction	Amount for 10 reactions (with 10% excess)
Reaction mix	19.9 µl	218.9 µl
Taq polymerase	0.1 µl	1.1 µl
Total volume	20 µl	220 µl

2.2.10 Preparation of real-time PCR reactions

The reactions were prepared by placing 20 µl of master mix into a PCR reaction tube and adding 5 µl of sample DNA. For the positive control, 5 µl of positive control reagent was added and the negative control consisted of master mix. All tubes were centrifuged at low speed to collect the contents to the bottom of the tube and then placed in the real-time PCR instrument for PCR according to the appropriate cycling conditions.

2.2.11 Fluorescence measurements

All the fluorescence measurements for the fluorescein labelled aptamers, the complementary oligonucleotides and the aptamer beacon were performed using a Nanodrop ND3300 fluorospectrometer (Thermo Scientific, Canada). The samples were excited in blue light (480 nm) and the emission was monitored at 515 nm. All the measurements were recorded in PBS buffer (pH, 7.4) at room temperature.

2.2.12 Designing aptamer sequences

The 54 nucleotide anti-*S. entirica serovar enteritidis* aptamer sequence was selected from a previously published study (**Kolovskaya** *et al.,* 2013). The truncation of this aptamer was based on the mfold software secondary structure. The on and off fluorescence assays of duplex and aptamer beacon were designed to determine the short sequence binding region of the aptamer. As shown in Table 1, three different designs were used.

In the first design, two aptamer beacons at the 5' and 3' ends (SE54MB1 and SE54MB2) of the full length aptamer were achieved by the addition or deletion of nucleotides from either the 5'or 3' end. The aptamer beacons were labelled with fluorescein and quencher (BHQ1) at the 5'and 3'ends of the stem, respectively.

The second design was the 29 mer sequence from the middle of the aptamer which contains part of both apta-beacons (SE54T). This design was used for the competitive displacement of aptamer complementary sequences (SE54TC1 and SE54TC2) and labelled with fluorescein and BHQ1 at 5' and 3' ends, respectively. The fluorescein and quencher became in close proximity to each other on hybridization with the truncated aptamer.

The third design was the same 29 mer sequence as the second design, with the fluorescein label (SE54TF) for the Graphene oxide (GO) experiments.

2.2.13. Determination of the binding affinity of the truncated aptamer

After selecting the truncated aptamer that contained the binding region to the target, the dissociation constant (K_d) of that sequence (SE54T) was determined by fluorescence assay. Briefly, 10^6 CFU/ml of *S. entirica serovar enteritidis* cells were first incubated in a 96-well microtiter plate overnight. The plate was then washed with PBS to remove the excess unabsorbed cells. Different concentrations of fluorescein labelled SE54T aptamer solutions in 100 µl PBS buffer were heated to 95°C for 5 min, cooled to 4°C for 10 min, kept at room temperature for 10 min and then incubated with the adsorbed cells in the wells for 1 h. Then, the wells

were washed twice with 500 µl PBS, suspended in 100 µl PBS buffer solution and the fluorescence was measured. The saturation curve was plotted as the SE54T aptamer concentration versus fluorescence intensities and the K_d was calculated using non-linear regression fitting of the curve as shown in **Fig.** 2.5.

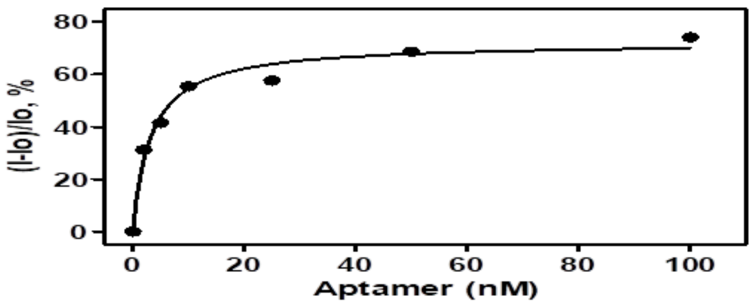


Fig. 2. 5: Binding affinity curve of the aptamer SE54T plotting the aptamer concentration versus the percentage change in the fluorescent intensity.

2.2.14. Aptabeacon and competitive fluorescence displacement assays

For the aptamer beacon assay, the aptamers were first heated to 95°C for 5 min, cooled to 4°C for 10 min and kept at room temperature for 10 min. Then, the *S. entirica serovar enteritidis* cells were incubated with 100 nM solutions of aptamer beacon and the fluorescence intensity of each sample was recorded. In the competitive fluorescence displacement assay, a mixture of 500 nM solutions of each truncated DNA aptamer and equal concentrations of their 5'fluoresceinlabelled and 3' BHQ1 labelled complementary sequences in PBS buffer were kept at 90°C in a water bath and then slowly cooled to room temperature for 3 hours. The slow cooling processes ensured a perfect duplex, bringing the fluorescein-BHQ1 pair as close as possible. Then, 100 nM of hybridized duplex was incubated with different concentrations of *S. entirica serovar*

enteritidis ranging from 10^2 to 10^7 CFU/ml for 30 minutes. The fluorescence intensity of each sample was recorded. The specificity of the SE54Tsequence was tested by incubating the duplex with 10^4 CFU/ml of similar bacteria associated with *S. entirica serovar enteritidis* such as St, *Ec* and Sa (see **Fig.** 2.6).

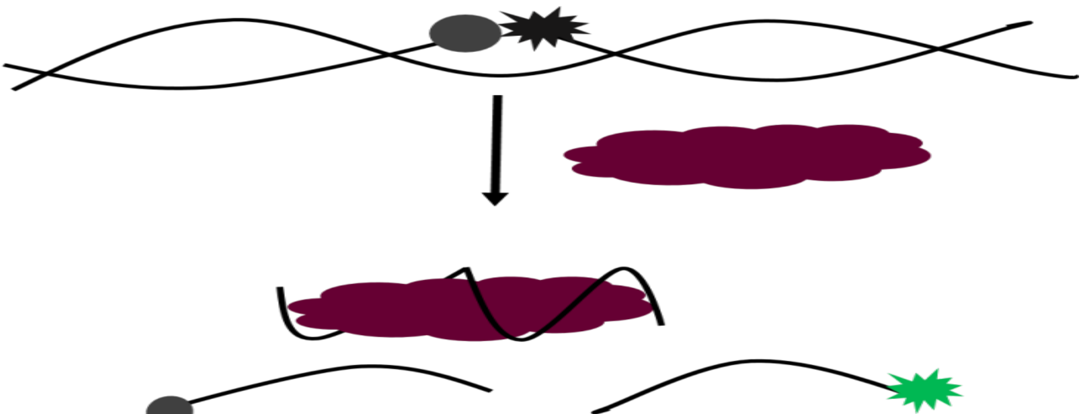


Fig. 2.6: Schematic diagram of the aptamer competitive displacement assay.

2.2.15. Confirmation of the bacterial count of serial concentrations by a standard counting method on agar media

2.2.15.1 Method

Four strains of *S. typhimurium, S. enteritidis, S. aureus* and *E. coli* were subcultured for 24 h in TSB. Then, a concentration of 10^8 was obtained from the mother culture by adjustment of the culture solution to 0.5 OD using a spectrophotometer at 660 nm. Each strain solution was then diluted from 10^8 to 10^1 using a sterile saline solution and 50 µl of culture was spread on 90 mm Petri plates containing specific media for each bacterium using INTERSCIENCE EASY SPIRAL® (*Salmonella* strains were cultured on *Salmonella* Chromogenic Agar, S. *aureus* was cultured on Baird Parker Agar and *E. coli* was cultured on Eosin Methylene Blue Agar). All the plates were incubated for 24 h at 37°C. Then, the plates were

counted and imaged using INTERSIENCE SCAN®. The plates reads are shown in the following **Table** 2.1.

Table 2.1: the bacterial count of samples plated out for confirming the used in screening assay.

Strain	Dilution	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8
S. enteritidis		>300	>300	>300	>300	>300	>300	360	33
S. typhimurium		>300	>300	>300	>300	>300	240	50	5
S. aureus		>300	>300	>300	>300	>300	>300	90	7
E. coli		>300	>300	>300	>300	>300	360	44	5

10^1	**10^2**	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**
>300	**>300**	**>300**	**>300**	**>300**	**360**	**44**	**5**

Fig. 2.7: Counts of the serial dilutions for S. entritidis in the range 10 to 10^8

10^1	**10^2**	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**
>300	>300	>300	>300	>300	**240**	**50**	**5**

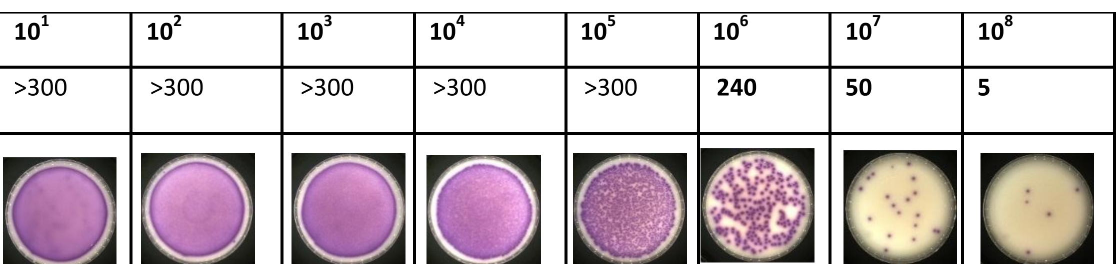


Fig. 2.8: Counts of the serial dilutions for S. entirica serovar typhimurium in the range 10 to 10^8

10^1	**10^2**	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**
>300	**>300**	**>300**	**>300**	**>300**	**>300**	**90**	**7**

Fig. 2.9: Counts of the serial dilutions for S. aureus in the range 10 to 10^8

10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8
>300	>300	>300	>300	>300	360	44	5

Fig. *2.10: Counts of the serial dilutions for E. coli 0157:H7 the range (10 to 10^8).*

Chapter 3 Development of a rapid immuno-based nanosensor for the detection of pathogenic bacteria in poultry processing plants

3.1. Abstract

A simple and reliable colorimetric immune sensor was developed and evaluated as a novel and rapid screening platform for pathogenic bacteria in poultry processing plants. This nano-based immune technique was used for the detection of pathogens present on surfaces, for example, the surfaces of poultry processing plants such as glass, stainless-steel and chicken meat. The antibodies were conjugated to cotton swabs, which were used for pre-concentration of the cells from contaminated surfaces. Then, the cotton swab was immersed in the same antibody conjugated with coloured nanobeads. Specific antibodies for each of the following bacteria: *Salmonella typhimurium, Salmonella enteritidis, Staphylococcus aureus and Campylobacter jejuni* were immobilized on different coloured beads and four different coloured beads for the targeted foodborne pathogens were used. The bacterial cells were sandwiched between the antibodies on the coloured beads and primary antibodies immobilized on the cotton swab. The assay was tested with different concentrations (10^1-10^8 CFU/ml) of *S. typhimurium, S. enteritidis, S. aureus* and *C. jejuni,* with the intensity of the coloured nanobeads on the cotton swab being proportional to the concentration of captured bacterial cells and a detection limit of 10 CFU/ml. Furthermore, specificity assays were performed by incubating other bacteria with the immobilized specific antibody for specific bacteria. The results showed that the sensors are high selective for the targeted pathogens. The proposed colorimetric assay has the potential for application for the rapid qualitative and semi-quantitative detection of microorganisms on surfaces in food processing plants.

3.2. Introduction

Different methods using various sensing principles have been used for the detection of pathogenic bacteria causing foodborne diseases (**Poltronieri** *et al.,* 2014). Conventional methods include culture-dependent methods, microscopy, PCR and other amplification techniques. Magnetic-immunoassays based on enzymatic absorbance and fluorescent signals have also been used for the detection of *Bacillus globigii* spores and cells (**Farrell** *et al*., 2005).

Recent trends in food technology and the increasing interest in food safety and quality improvement, has led to the development of new rapid detection methods for food contamination (**Rohde** *et al*., 2015). Such methods should be simple, low cost, and portable, giving precise results in the shortest time. In this paper, a novel assay was developed utilizing a nanoparticle-based immunoassay. The assay was used for the detection of *Salmonella* strains, *Staphylococcus aureus* and *Campylobacter jejuni* on the surfaces of poultry processing plants. In this method, the cotton swap was functionalized with aldehyde groups to immobilize the recognition receptor, such as antibodies, for the detection of bacterial stains. Then, the cotton swab was immersed in a cocktail of coloured nanobeads with various specific antibodies to develop a rapid, sensitive and selective colorimetric assay for the detection of pathogenic bacteria on the different surfaces.

3.2.1. Cotton as a capturing substrate

3.2.1.1. Modification of cellulose (cotton surface)

Cellulose is the most abundant and important biopolymer present in nature and is the basic structural component of plant cell walls. Cellulose is a complex carbohydrate or polysaccharide with the formula $(C_6H_{10}O_5)_n$ (**Wade,** 1999). It consists of a linear chain of hundreds to thousands of β-(1, 4)-D-glucose molecules as shown in **Scheme** 3.1. Cellulose is used in many different industries, such as textile, pharmaceuticals, energy drinks and explosive cellulose (***www.bio.plaisley.ac.uk***). It can be further modified by various chemical derivatizations such as oxidation, esterification, and silylation resulting in functionalized microfibres for various applications.

Scheme 3.1: Structure of cellulose

In oxidizing media, cellulose can undergo many modifications and accordingly, oxidized cellulose or oxy-celluloses have different properties (***Varavinit*** *et al.,* 2001). Attacks on cellulose by oxidizing agents are mainly directed to three positions on the cellulose molecule (**Rutherford** *et al.,* 1942). The first position is the primary alcohol group which can be oxidized into aldehyde or carboxyl groups, the second position is the

aldehyde end-group which can be oxidized to a carboxyl group, and the third position is the glycol group which can be modified into a ketone, aldehyde or carboxyl group. It has been assumed that the oxidative attack is principally targeted at the primary alcohol group (**Rutherford** *et al.,* 1942). The periodate oxidation of cellulose in sulphuric acid is one of the common oxidizing methods characterized by a specific cleavage of the C2-C3 bond of the residue. It can produce dialdehyde cellulose in which the 1, 2-glycol groups of the glucose residues are converted into two dialdehyde groups**.**

3.2.1.2. Immobilization of antibody on the cotton surface

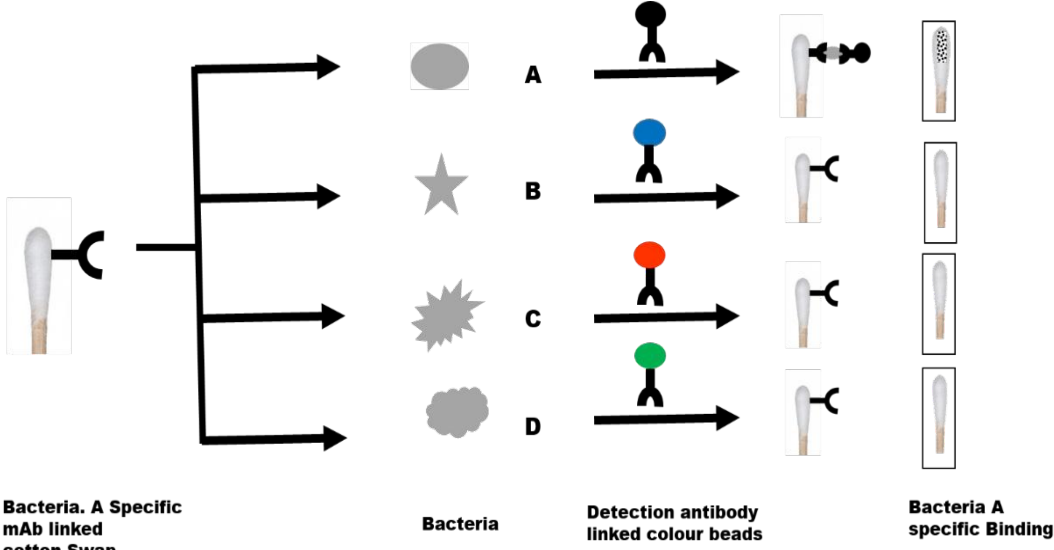
Preparing the biorecognition surface is an important and essential step in fabricating biosensors. Therefore, detection with high analyte specificity and binding strength is crucial for highly accurate results. Antibodies (Abs) are the most preferred detection elements that have been increasingly used in biosensors, but immobilization of these antibodies on the biosensor surface remains a key challenge in this process (**Isobe** *et al.,* 2011). Many strategies have been applied for the immobilization of Abs to improve biosensor performance and the choice of immobilization method is based on how to achieve it without affecting the binding activity and specificity of the Abs. Generally, the immobilization process comprises three steps, in the first step, a film containing a functional group (carboxyl, aldehyde, amine) is created. On the biosensor surface, the second step involves activating functional groups with cross-linkers such as a mixture of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and NHS. The third step

involves the reaction of the recognition element with the linkage agents (**Trilling** *et al.,* 2013).

3.3. Results and Discussion

3.3.1. Detection of pathogenic bacteria

Pathogenic bacteria such as *S. entirica serovar typhimurium* (St), *S. entirica serovar enteritidis* (Se), *S. aureus* (Sa)and *C. jejuni* (Cj) were screened using a sandwich assay, where the pathogenic bacteria were sandwiched between the primary antibody immobilized on cotton swab surface and the secondary antibody conjugated on coloured nanobeads as shown in the **Scheme** 3.2. The target bacteria cells were pre-concentrated by capturing the cells from the surfaces by primary antibodies immobilized on cotton swabs, form primary antibody-cell (Cotton-C-mAb-cell) complexes due to antigen-antibody interactions. In the next step, the secondary antibody conjugated with coloured nanobeads bound to the cells captured by the primary antibody. As the secondary antibodies are specific to the target cells, only one coloured nanobead binds to the captured cell and the cotton surface turns the colour of the beads. The confirmation of binding of each bacterium with their corresponding specific antibody was confirmed by real-time PCR.



Scheme 3.2. Schematic diagram of selective binding of pathogenic bacteria. The capture mAb conjugated cotton incubated with four different bacterial stains (A, B, C and D) to preconcentrate the target cells. In the next step, all the pre-concentrated stains were immersed by target (A) specific antibody conjugated beads. The colour of the cotton surface indicates the presence of that pathogen (A).

3.3.2. *Salmonella typhimurium* assay

The cotton swabs linked with *S. typhimurium*, St specific capture antibody and StC-mAb were swabbed over surfaces of chicken meat, glass slides and stainlesssteel surfaces spiked with St. St-mAb in the cotton swabs formed StC-mAb-St complexes and as the bacterial cells are bigger in size than the antibody molecules, there were a greater number of antibody binding receptor sites on the cell surface. This complex was further treated with a solution of secondary detection antibody (StD-mAb) linked magnetic nanoparticles, forming a black St sandwich complex, Cotton-StC-mAb-cell-mAb-StD as shown in **Fig.** 3.1 A, B and C for chicken meat, glass slide and stainless-steel surfaces respectively. The unbound

magnetic beads were collected by passing the cotton swab close to permanent magnet sheet. Using the magnetic beads has an advantage over polymer nanobeads by eliminating the washing steps. To determine the detection limit of the assay, StC-mAb linked cotton was swabbed over three surfaces, chicken meat, glass slide and stainless-steel, artificially contaminated with various concentrations (10 to 10^8 CFU/ml) of St cells, followed by sandwich complexation. The intensity of the black colour on the cotton surface increases proportionately with the increasing concentration of cells for all the samples as shown in the **Fig.** 3.1 A, B, and C. When the cotton swab was treated with a high concentration of bacteria, more cells were captured by the capture antibodies, StC-mAbs. In the second step, more magnetic beads form sandwich complexes due to the availability of abundant binding sites on the cell surfaces. The high intensity of the black colour is explained by the formation of more *St* sandwich complexes on the cotton surface at higher cell concentrations. It is a simple technique in which the pathogenic bacteria on the contaminated surfaces of the meat processing plant can be easily identified from the colour of the cotton surface by the naked eye, as shown in **Fig.** 3.1 A, B, and C. However, the visual detection limit varies with the concentration of the bacterial cells in the sample. In this experiment, the visual detection limit of *S. entirica serovar typhimurium* from the chicken meat and the stainless-steel plates was 10^1 CFU/ml, whereas, the sample from the glass plate was 10^2 CFU/ml, as shown in **Fig.** 3.1 A, B, and C respectively. The higher detection limit for the glass slide might be due to the strong physical adsorption of *S. entirica serovar typhimurium* bacterial cells to the surface of the glass plates. The

intensity of colour as a function of the *S. entirica serovar typhimurium* concentrations on chicken, glass plate, and stainless-steel surfaces is shown in **Fig.** 3.7. The detection limits of the chicken and stainless-steel surfaces are comparable with previous results using immunomagnetic nanospheres and immunofluorescent nanospheres, 10 CFU/ml (**Wen** *et al.*,2013). However, our method is very simple, instrument-free and can be used for on-site detection. The relationship between cell number of bacteria and colour intensity on the cotton swabs indicates that this method can be used, not only for qualitative, but also for semi-quantitative analysis.

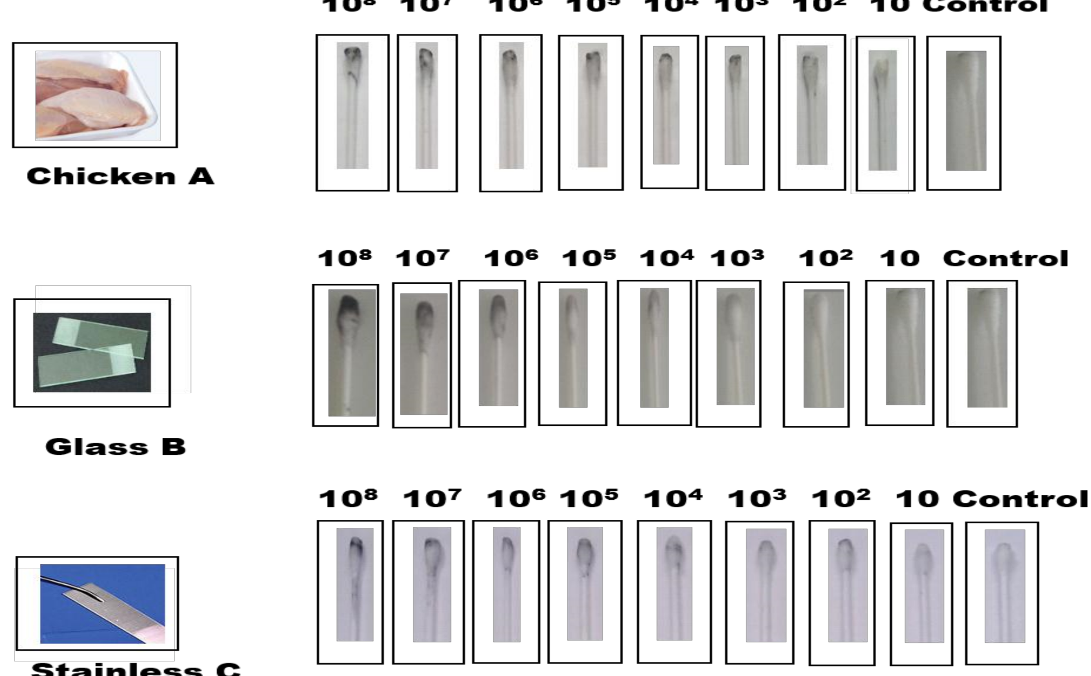


Fig. 3.1 A: *Salmonella typhimurium* screening. *S. entirica serovar typhimurium* specific antibody conjugated cotton swab and the black magnetic beads captured the *S. entirica serovar typhimurium* target cells from chicken (A), glass plate (B) and stainless-steel surfaces (C) by sandwich binding. More intense black sandwich complexes were formed with the increasing concentration of bacterial cells. The visual detection limit for the samples from chicken, glass and stainless-steel are 10^1, 10^3 and 10^1 CFU/ml respectively.

3.3.3. *Salmonella enteritidis* assay

This assay was the same as for *S. typhimurium, with only one change that was in using Salmonella enteritidis* (Se) specific antibodies for the capture and detection steps. The antibody sandwich bacteria cell complex, SeC-mAb-cell-mAb-DSe, was formed by treating the cotton swab with secondary detection antibody conjugated blue polymer nanobeads, SeD-mAb. The results of the detection of Se on chicken, glass slides and stainless-steel surfaces are shown in **Fig.** 3.1 A, B, and C. **Fig**. 3.2 shows that the intensity of the blue colour on the cotton surface increases with increasing bacterial cell counts on the three tested surfaces. The visual detection limit of *S. entirica serovar enteritidis* on chicken, glass and stainless-steel surfaces were 10^2, 10^1 and 10^1 CFU/ml respectively. Compared to the previously reported fluorescence resonance energy transfer (FRET) based detection of *S. entirica serovar enteritidis* in milk and water (10^2 to 10^3 CFU/ml; **Duan** *et al.*, 2016), this cotton swab based method had a lower detection limit, was more sensitive for on-site detection and instrument-free. The high detection limit for the sample from glass is probably due to the strong adsorption between the cell and the glass surface. The colour intensity as a function of the *S. entirica serovar enteritidis* concentrations on chicken, glass plate, and stainless-steel surfaces is shown in **Fig.** 3.8.

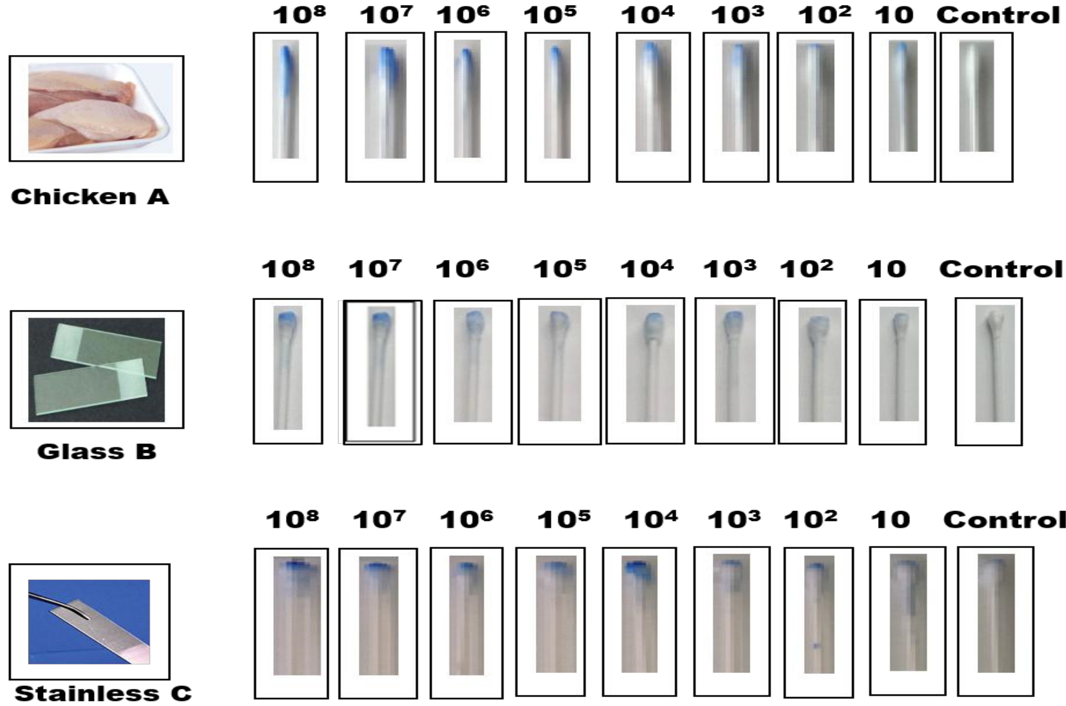


Fig. 3.2: ***Salmonella enteritidis screening*****:** ***S. entirica serovar enteritidis*** **specific antibody conjugated cotton swabs and the blue polymer beads captured the** ***S. entirica serovar enteritidis*** **target cells from chicken (A), glass plate (B) and stainless-steel surface (C) by sandwich binding. More intense blue sandwich complexes were formed with the increasing concentration of bacterial cells. The visual detection limit for the samples from chicken, glass and stainless-steel are 10^1, 10^3 and 10^1 CFU/ml respectively.**

3.3.4. *Staphylococcus aureus* assay

For *Staphylococcus aureus* (SA) detection, Sa binding antibodies were used in the capture and detection steps. ScC-mAb-cell-mAb-DSc sandwich complexes were formed by antibody conjugated orange nanobeads when treated with serial dilutions of cells in the range of 10 to 10^8 CFU/ml. The intensity of the orange colour on the cotton surface was proportional to the cell counts, as shown in **Fig.** 3.3 A, B and C, explaining the formation of increasing amount of Sa sandwich complexes

on the cotton surface at higher cell counts. The visual detection limit of *S. aureus* on all three surfaces was 10^1 CFU/ml (**Fig.** 3.3 A, B and C). The low detection limits indicate the easy availability of cells for complex formation on all surfaces, as a polyclonal antibody was used in this case. (Zelada-**Guillén** *et al* 2013) developed a biosensor for the detection of *S. aureus* on the skin using anti-*S. aureus* DNA aptamer as a recognition receptor and single-walled carbon nanotubes (SWCNTs) as an ion-to-electron potentiometric transducer. The sensor had a high detection limit (10^3 CFU/ml) [43] compared to the new method described in this study. Moreover, the newly developed method has a low detection limit and as it is colorimetric, the results can be observed by the naked eye.

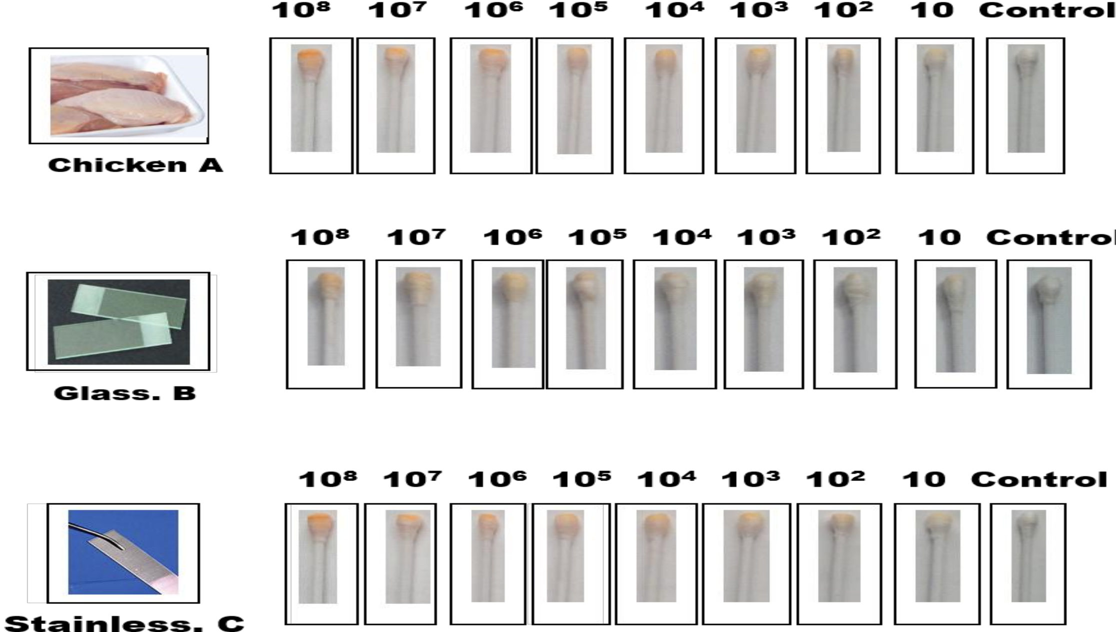


Fig. 3.3: Screening *Staphylococcus aureus*: *S. aureus* specific antibody conjugated cotton swab and the orange polymer beads captured the *S. aureus* target cells from chicken (A), glass plate (B) and stainless-steel surface (C) by sandwich binding. The more intense orange colour sandwich complexes were formed with the increasing concentration of bacterial cells. The visual detection limit for the samples from chicken, glass and stainless-steel are 10^1, 10^3 and 10^1 CFU/ml respectively.

3.3.5. *Campylobacter jejuni* assay

Green polymer nanobeads linked with *Campylobacter jejuni* binding antibody were used as a marker for *Campylobacter jejuni* (Cj) screening, with a Cj selective binding antibody used for the to capture and target detection steps. Green sandwich (CjC-mAb-mAb-CjD) complexes were formed on the cotton surfaces on treatment with serial dilutions of cells. As shown in **Fig.** 3.4 A, B and C, the intensity of the green colour increased with the increasing cell counts in the sample. An intense colour of the cotton surface was observed, even at a low concentration (10 CFU/ml) for the samples from stainless-steel surface, whereas, the intense colour was only observed at elevated concentrations (10^2 CFU/ml) on chicken and glass plates, presumably due to the weak interaction between the sample surface and *Campylobacter*. The intensity of colour in relation to the *C. jejuni* concentrations on chicken, glass plate, and stainlesssteel surfaces is shown in **Fig.** 3.4. Recently, a quartz crystal microbalance (QCM) based-sensor for the detection of *C. jejuni* was reported to have a sensitivity of 150 CFU/ml (**Masdor** *et al.,* 2016), which was comparable to our detection limit. However, our method has many advantages over this method and is simpler for the on-site detection of *C. jejuni*.

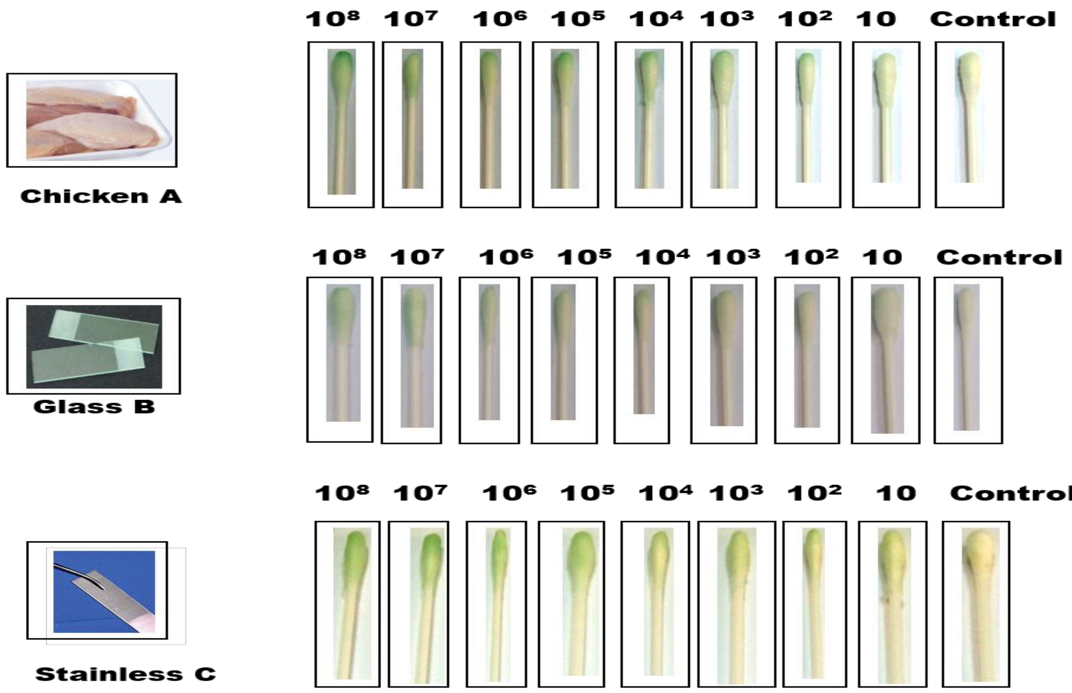


Fig. 3.4: *Campylobacter jejuni* screening: *C. jejuni* specific antibody conjugated cotton swab and the green polymer beads captured *C. jejuni target* cells from chicken (A), glass plate (B) and stainless-steel surfaces (C) by sandwich binding. More intense green sandwich complexes were formed with the increasing concentration of bacterial cells. The visual detection limit for the samples from chicken and glass are 10^2 CFU/ ml and for stainlesssteel, 10^1 CFU/ml respectively.

3.3.6. Specificity assays

Specificity is a major indicator of the success of the sensor performance, therefore, the recognition receptors used in sensor development must be tested for specificity. The antibodies used in the recent study were tested for their specificities and **Fig.** 3.5 summarizes the binding assays for the four targeted antibodies. The specific binding of the *S. entirica serovar typhimurium* antibody was tested using four different pathogenic bacterial cells, *S. typhimurium*, *S. aureus, E. coli* and *C. jejuni*. Four cotton swabs conjugated with *S. entirica serovar typhimurium* antibody were

used for individually swabbing the four surfaces artificially contaminated with St, Se, Sa and Cj. Each cotton swab was further incubated with a developing solution, which contained nanobeads with different colours, with each colour specific for the antibody of the various targeted bacteria. After extensive washing, the black magnetic beads on cotton swabs treated with Se, Sa and Cj were washed away, and only the cotton swabs treated with *S. entirica serovar typhimurium* retained their magnetic beads on the surfaces. The black colour sandwich complex on the cotton swab confirmed the specific binding of *S. entirica serovar typhimurium* from the mixture of bacterial cells as shown in **Fig.** 3.5 A.

The specific detection of *S. entirica serovar enteritidis* was tested by the same method used for *S. entirica serovar typhimurium* except for the use of *S. entirica serovar enteritidis* specific capture and detector antibodies. Blue colour beads conjugated with *S. entirica serovar enteritidis* specific binding antibody were used in the detection step. After extensive washing, only blue colour beads adhered to the cotton surface, indicating the specific binding of *S. entirica serovar enteritidis* as shown in **Fig.** 3.5 B. The results for specificity test for the specific antibodies for *S. aureus* are shown in **Fig.** 3.5 C. In this assay, the same method was used for *S. typhimurium,* but the orange colour of the *S. aureus* binding antibody linked cotton after treatment with a mixture of bacterial cells confirmed the specific binding of *S. aureus.* The specific binding of *C. jejuni* from the mixture of four different pathogenic bacteria was demonstrated by the formation of a green colour sandwich immunocomplex on the cotton surface in **Fig.** 3.5 D.

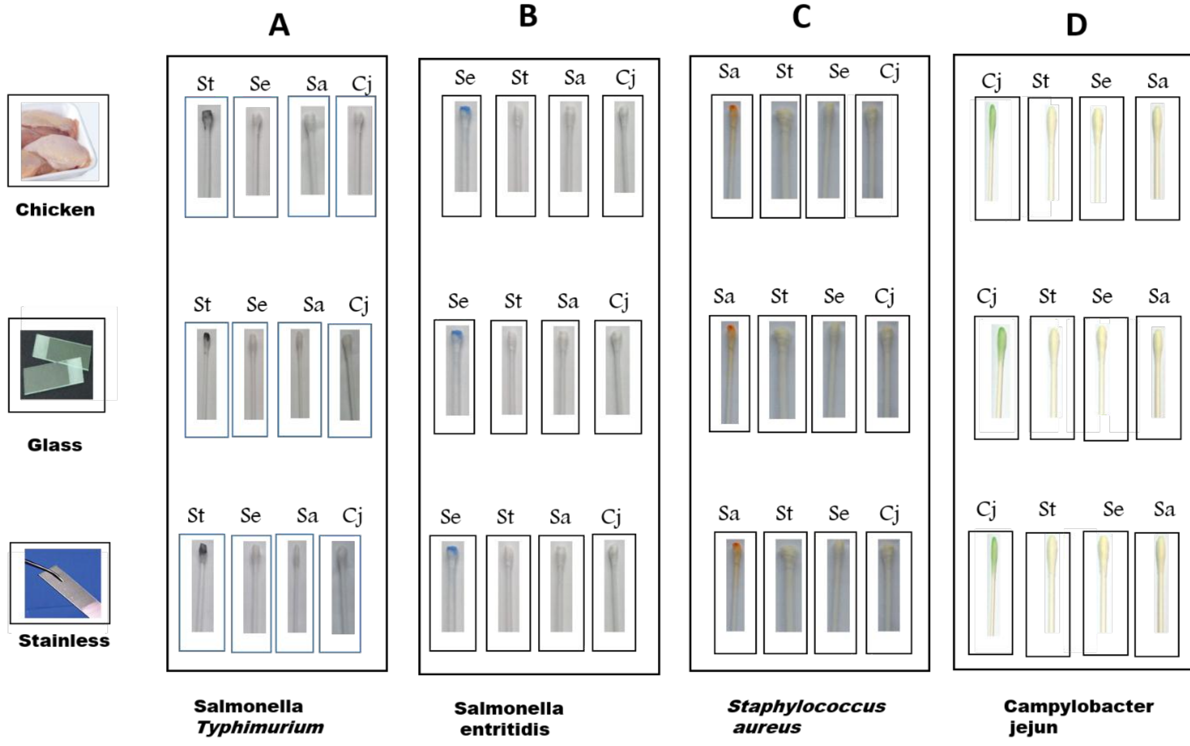
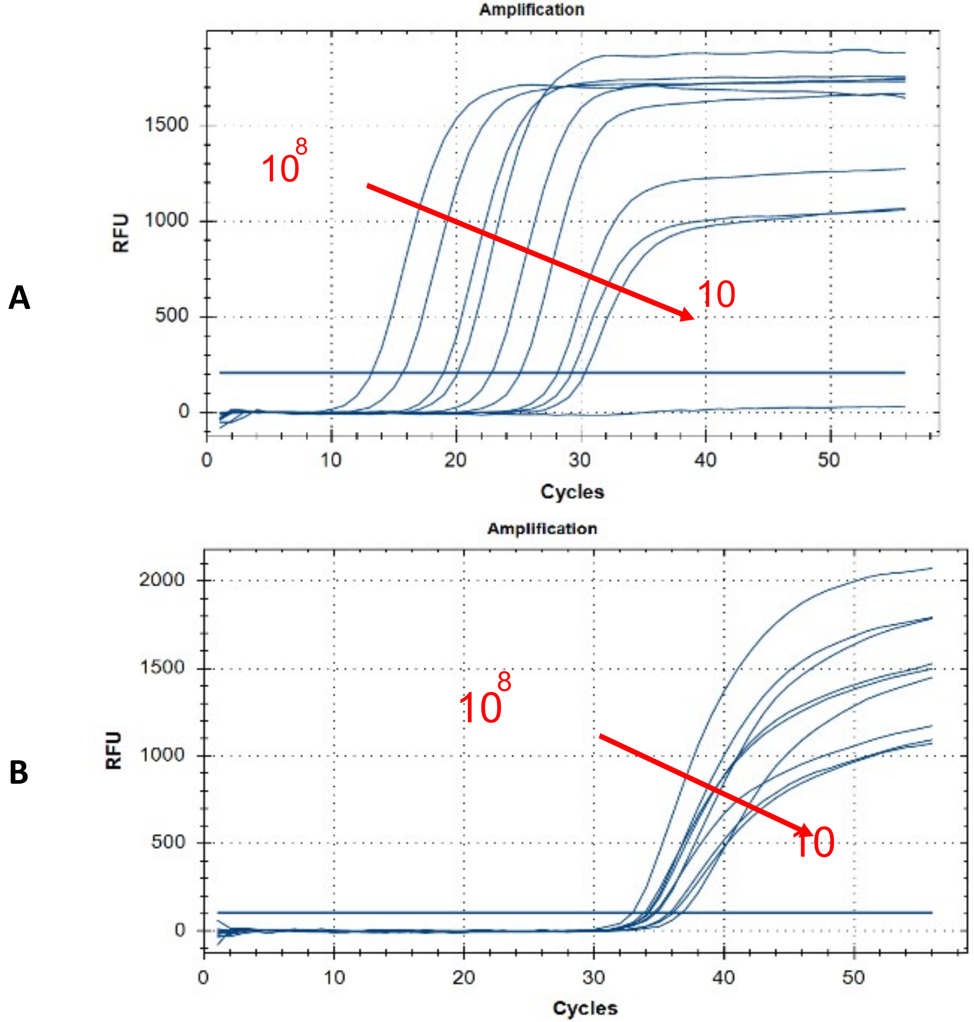


Fig. 3.5: Evaluation of specificity of binding: (A) *S. entirica serovar typhimurium* specific capture mAb conjugated cotton incubated with *S.* enteritidis, *S. aureus* and *C.* jejuni to preconcentrate the target cells. The pre-concentrated stains were immersed in *S. entirica serovar typhimurium* specific detection antibody conjugated magnetic beads. The black magnetic beads selectively bind with *S. entirica serovar typhimurium* treated cotton and turn black. (B) Similarly, the *S. entirica serovar enteritidis* specific antibody conjugated beads turned blue indicating the specificity for *S.* enteritidis. (C) The *S. aureus* specific antibody conjugated beads turned orange indicating the specificity for *S. aureus and* (D) *C. jejuni* specific antibody conjugated beads turned green indicating the specificity for the detection of *C. jejuni*.

3.3.7. PCR confirmation assays

The presence of the target pathogens *Salmonella spp., S. aureus and C. jejuni* on the coloured cotton surfaces was further confirmed by real-time PCR as shown in **Fig.** 3.6. There was an increase in DNA amplification (see **Fig**. 3.6 A, B, C and D) with the increasing cell counts of *Salmonella spp., S. aureus and C. jejuni* from 10 to 10^8 CFU/ml treated stainless-steel surface samples as illustrated in **Fig**. 3.7A, B, and C respectively.



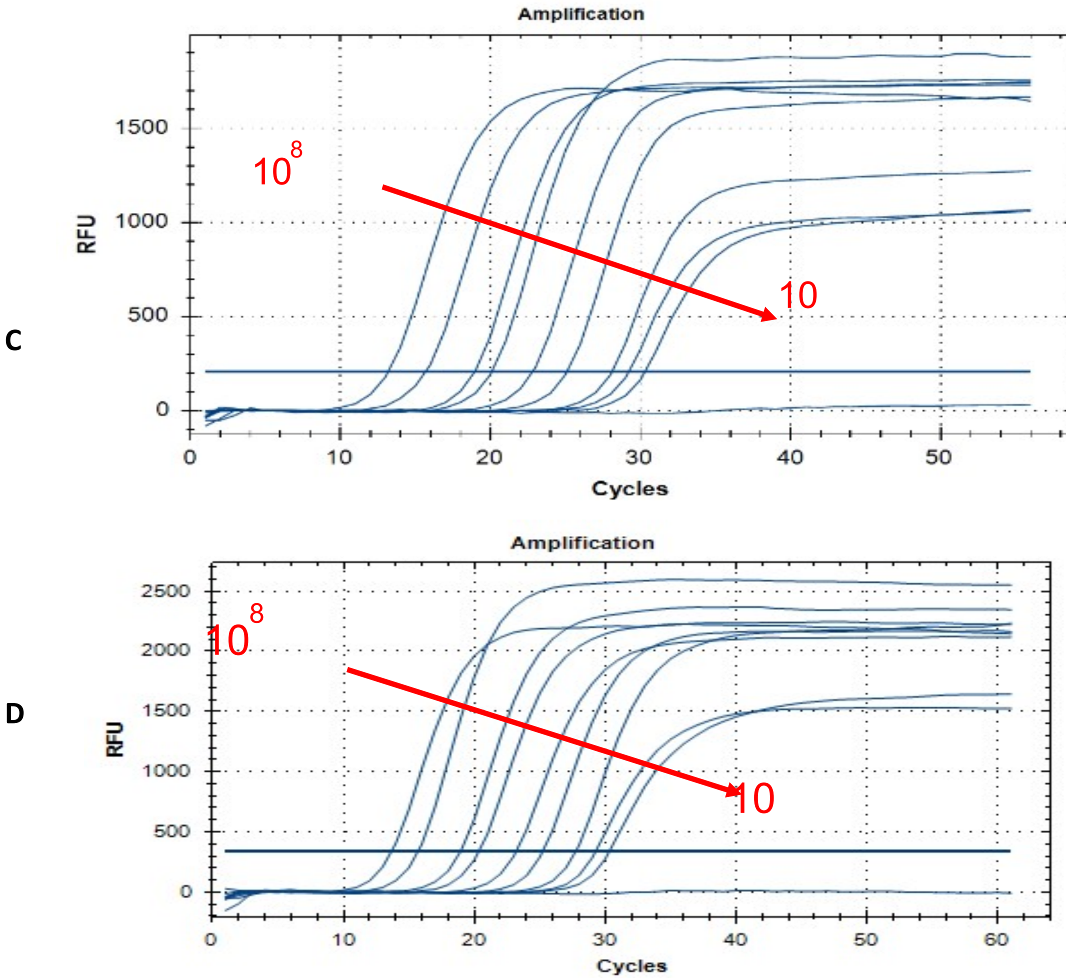


Fig. 3.6: Real-time PCR confirmation of the presence of 10 to 10^8 CFU/ml of *Salmonella spp.* (A & B), *S. aureus* (C) and *C. jejuni* (D) on the coloured cotton surfaces collected from contaminated stainless-steel plates.

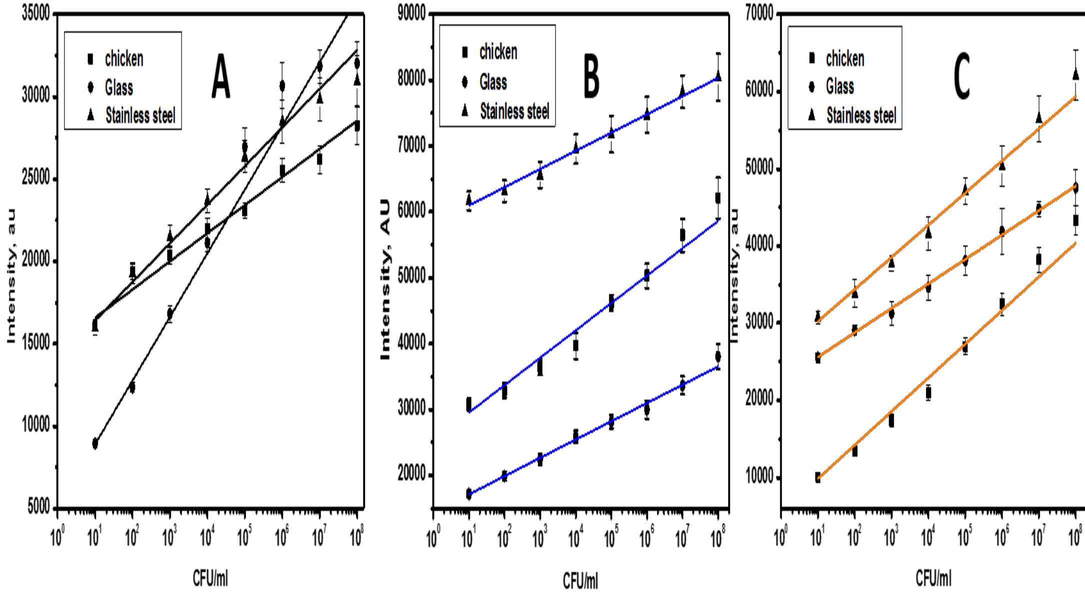


Fig 3.7: The intensity of colour as a function of concentration on the three surfaces (chicken, glass and stainless steel). (A) Salmonella entritidis (B) salmonella typhimurium, (C) Campylobacter jejuni.

3.4. Comparison of the recent assay with previous studies

By comparing the results of the current study with those obtained from a number of previous studies, in terms of methodologies and sensitivities, we can identify two main advantages to the more recent assay. The first is, the innovative way which was used to make the sandwich immunoassay, which results in the procedure being faster and more cost-effective. This is because using cheap materials like cotton enhances the simplicity of the assay and reduces the need for instruments and tools. The second reason is that the high sensitivity of the assay, represented by the low limit for the detection of all bacteria to be assayed. In the following table, we show a comparison between this latest technique and some from previous studies.

Table 3.1. Comparison between recent study and some previous methods in terms of sensitivity, cost and simplicity.

Ser.	Bacteria	Nanoparticle	Detection method	LOD (CFU/ml)	Adv/disadvantage	Reference
1	*Salmonella*	Superparamagnetic Fe3O4 nanoparticles	Immuno-magnetic separation	100	Rapid and cost effective	(Joo j. et al. 2012)
2	*Escherichia coli and staphylococcus aureus*	Polyethyleneimine (PEI)-modified Au-coated magnetic microspheres (Fe3O4@Au@PEI)	SERS detection method	100	Simple operating procedure, total assay time 10 min.	(Wang 2016)
3	*Escherichia coli and staphylococcus aureus*	FePt@Van magnetic nanoparticles	Fluorescence microscopy	4	Bacteria detection in 2 hours.	(Gao 2006)
4	*Escherichia coli*	Cysteine gold nanoparticles (CAuNPs)	Colorimetric method	100	Fast, visual method	
5	*Escherichia coli Salmonella Vibrio cholera Campylobacter jejuni*	Streptavidin coated magnetic nanoparticles	Multiplex PCR	100	Simultaneous detection of four pathogens	(Li 2013)

6	*Campylobacter jejuni*	Bimetallic Au@Ag core–shell structures	Immuno-magnetic separation polymerase chain reaction (IMS-PCR) method	100	Cost-effective, only basic equipment needed	(Cao 2011)
7	*Escherichia coli*	Silver NPs (AgNPs)	Anodic particle coulometry technique		Single bacteria detection	(Sepunaru 2015)
8	*staphylococcus aureus*	Au-coated magnetic nanoparticles (AuMNPs)	SERS detection method	10	Low limit of detection	
9	*Salmonella*	Gold nanoparticles (AuNPs)	Electrochemical detection method	143		(Andre Afonso et al. 2013)
10	*Salmonella*	Chitosan-gold nanoparticles	Electrochemical detection method	5	Low LOD but, need instrument.	(Cuili Xiang et al. 2015)
11	*Listeria monocytogenes*	Magnetic nanoparticles.	Colorimetric	217	Simple with high LOD.	(Sahar al hogail et al. 2016)
12	*Staphylococcus aureus*	Magnetic nanoparticles	Paper-based biosensor	100	Simple with good LOD	(Chadeer et al. 2017)
14	*Salmonella typhimurium*	Colorimetric aptasensor	UV visible spectrophotometer	56	Need instrument	(Xiaoyuan et al. 2017)
15	*Salmonella entritidis*	DNA –aptamer colorimetric	Capillary detection platform.	1000	Low LOD	(Ceren Bayraç et al. 2017)

16	*Brucella spp.*	Quantum dots	Fluorescence spectrophotometer	1000	Low LOD	(Dandan song et al. 2017)
17	*Staphylococcus aureus*	Gold nanoparticles	Colorimetric aptasensor using plate reader	9	Low LOD, need instrument.	(Jinglei yuan et al. 2014)

3.5. Conclusion

A simple, rapid, low cost assay for the detection of pathogenic bacteria on the surfaces of poultry processing plant was developed. The type of bacteria present on the contaminated food was identified by the colour formation on the cotton surface. In the newly developed nano-based biosensor assay, a specific antibody immobilized cotton swab was used for pre-concentrating the pathogens, then complexed with antibody conjugated on coloured nanobeads. The successful detection of *S. typhimurium, S. enteritidis*, *S. aureus* and *C. jejuni* was demonstrated by the formation of various colours on the cotton surfaces. Furthermore, the colour intensity was proportional to the concentration of target pathogens. This relationship between the bacterial cell number and colour intensity indicated that this method can be used for qualitative as well as semi-quantitative detection. The detection limits for the assay were as low as 10 CFU/ml, comparable with the recent reported detection limits, indicating the method’s sensitivity. Moreover, the assay can be carried out by non-skilled personnel by the naked eye and is instrument-free. The specificity of the assays was also confirmed by real-time PCR. In conclusion, the developed biosensor has the potential for

the on-site rapid screening of bacterial contamination in food products (chicken, ready to eat foods, ground meat) as well as other biomedical, environmental and security applications.

Chapter 4 Rapid colorimetric immunoassay for the detection of foodborne pathogenic bacteria in poultry processing plants

4.1. Abstract

A rapid method for the detection of pathogenic bacteria, *Salmonella typhimurium, Salmonella enteritidis, Staphylococcus aureus* and *Campylobacter jejuni* was developed using a colorimetric immuno-sensor. The method is suitable for the on-site detection of pathogenic bacteria on the surfaces of chicken meat. The assay used activated cotton swab coupled with lactoferrin, which is used for pre-concentrating pathogenic bacteria from the contaminated chicken surfaces. The pre-concentrated cotton swab was immersed in the developing solution, consisting of different coloured polymer nanobeads immobilized with the *S. typhimurium, S. enteritidis, S. aureus and C. jejuni* specific antibodies. The coloured beads form sandwich complexes with the pathogenic bacteria, resulting in a change in the colour of the cotton surface. Each colour represents the presence of the corresponding pathogenic bacteria and the intensity of the colour on the cotton surfaces increased with the increasing the concentration of pathogenic bacteria. The detection limit was as low as 10 CFU/ml for *S. enteritidis*, 100 CFU/ml for *S. entirica serovar typhimurium* and *C. jejuni* and 1000 CFU/ml for *S. aureus.* This method is highly specific and its specificity was further confirmed by LAMP. This new colorimetric immuno-assay is a promising method for the detection of pathogenic bacteria on chicken and has the potential for application in healthcare, food, agriculture, environment and biodefence.

4.2. Introduction

An increase in the consumption of street foods, contaminated drinking water and preparation of ready to eat foods without proper safety measures leads to food safety issues. The detection and control of foodborne pathogens are important to protect the public from foodborne illness. Several conventional sensitive methods have been developed for the detection of foodborne pathogens. However, these methods are time consuming, require well experienced technicians and are expensive. To protect public health, disease spread must be controlled, therefore, there is a demand for a rapid and sensitive method for the detection of foodborne pathogens.

Several advances in methods have been developed based on various principles (**Mandal** *et al.,* 2011; **Poltronieri** *et al.,* 2014; **Zhao** *et al.,* 2014). The ELISA is one of the rapid pathogen detection immunological methods relying only on the recognition of the antibodies (Abs). However, they have limitations such as cross reactivity of the polyclonal antibody, high cost for antibody production, preprocessing and poor detection limit (**Lee** *et al.,* 2014). PCR is widely used for the detection of pathogens using specific primers and real-time PCR (RT-PCR) quantifies pathogens using DNA intercalating fluorescent dyes (**Law** *et al.,* 2014; **Zhao** *et al.,* 2014). A multiplex PCR (mPCR) approach has been used for the detection of more than one pathogen simultaneously (**Law** *et al.,* 2014; **Lee** *et al.,* 2014; **Van Giau** *et al.,* 2015). Indeed, **Chen** *et al.* (2012) detected five pathogens simultaneously using mPCR. In addition, more sophisticated analytical methods, such as liquid/gas chromatography coupled with

mass spectrophotometer, have been used for the analysis of pathogens. Although they are accurate and sophisticated, they are not suitable for use at the pointof-care on-site pathogen detection, that is because of large size of analysis instruments and complexity of multistep sample preparation methods which make it very difficult to be transported to the site of care. In addition to the high cost of these instruments.

Lactoferrin (LF) or lactotranferrin is a globular glycoprotein which binds iron, DNA, RNA, polysaccharides, heparin, bacteria, proteins, and viruses etc. Lactoferrin molecules are composed of a polypeptide chain with 703 amino acids forming two globular lobes, each lobe has one iron binding site. It is mainly produced from saliva, milk and exocrine secretions (**Masson** *et al.,* 1966), with high concentrations of lactoferrin found in human colostrum. Several studies refer to the ability of lactoferrin to bind most bacterial cells, suggesting that the binding between lactoferrin and bacteria could be caused by an electrostatic interactions (**Shi** *et al.,* 2000).

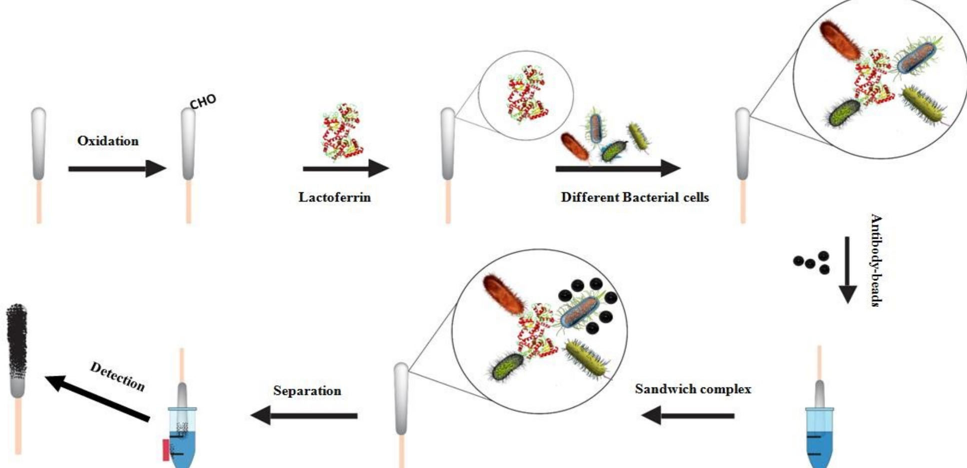
As it binds bacterial cells, lactoferrin was used for the development of a new simple low cost, portable and rapid sensor for the detection of *S. typhimurium, S. enteritidis, S. aureus* and *C. jejuni* on chicken meat. In this work, lactoferrin was used as a general capturing reagent to pre-concentrate the pathogen from chicken. The pre-concentrated pathogen formed sandwich complexes with the pathogen specific antibody coupled to nanoparticle-based dye coated coloured polymer beads, thereby changing the colour of the cotton surface.

4.3. Screening procedure

The screening procedure consists of two steps, the first step was a bacterial capture step and the second step was the sandwich formation with the secondary antibody for colour development as shown in **Scheme** 4.1. In this study, lactoferrin (LF) was used as a universal recognition receptor. Lactoferrin, as the pre-concentrating agent was conjugated to the cotton swab and the magnetic beads or coloured polymeric nanobeads were conjugated to the secondary detection antibodies (D-mAb) used for the colour development. In the first step, the cotton immobilized lactoferrin was swabbed over the contaminated chicken surfaces to capture the bacteria. The chicken meat was contaminated with St, Se, Sa and Cj bacterial cells and the number of bacterial cells on each surface was confirmed by cell counting. The cotton immobilized lactoferrin-bacteria complexes were washed with PBS to remove free cells from the cotton.

In the second step (detection step), the bacteria were sandwiched between the lactoferrin and secondary detection antibody conjugated nanobeads. The cotton swab lactoferrin-bacteria (cotton-LF-cell) complexes were immersed in coloured polymeric nanobeads or magnetic nanobeads linked with the secondary antibody in PBS buffer for 2 min. The LF-cell-secondary antibody (LFcell-mAb-D) sandwich complex was washed with PBS to remove the unbound beads. The colour of the cotton swab indicated the specific bacterial strain present on the contaminated surfaces as shown in **Fig.** 4.1.

Specific detection of the target bacteria was tested by incubating the lactoferrin immobilized cotton swabbed over surfaces contaminated with the bacterial cells and washed with PBS. Then, the cotton swabs were immersed in four different secondary antibodies conjugated to coloured nanobeads for 2 min. Finally, the cotton swabs were washed with PBS to remove the unbound beads. In the case of magnetic beads, the unbound particles were separated by a small magnetic sheet passed over the cotton swab. The colour of the cotton surface matched the colour of the specific secondary antibody conjugated to the nanobeads and indicated the specific binding of the target bacteria.



Scheme 4.1: Schematic diagram of the immune-biosensor for screening pathogenic bacteria from contaminated chicken meat. The aldehyde functionalized cotton swab was linked with lactoferrin that binds the target bacteria, including other bacteria. The lactoferrin complex was then treated with a cocktail of different coloured beads (in which each coloured bead was linked with a specific antibody stain). The target pathogenic bacteria present in the contaminated food will be identified by the colour of the cotton surface after formation of the sandwich immunocomplexes.

4.4. Results and Discussion

This paper reports the detection of foodborne illness causing bacteria found on chicken meat. *S. entirica serovar typhimurium* (St), *S. entirica serovar enteritidis* (Se), *S. aureus* (Sa) and *C. jejuni* (Cj) are the more frequently reported pathogens causing foodborne illnesses, therefore, a simple sandwich immuno-based colorimetric method was developed for the detection of these bacterial strains using magnetic and polymeric coloured nanobeads. Although the sandwich method is a well-established method for the detection of pathogenic microorganisms, this newly developed method utilizes the same principle to make a simple, low cost, portable colorimetric device for the point-of-care applications using a cotton swab as the supporting matrix.

The cellulose cotton was oxidized to active aldehyde using an acidic periodate solution. The appearance of a new peak at 1725 cm^{-1} in the IR spectra of the functionalized cotton confirmed the presence of an active aldehyde group (**Zhang** *et al.,* 2014). Lactoferrin was used as a capturing and pre-concentration agent, as this glycoprotein binds to bacteria and virus surface proteins. The amine group of the lactoferrin was immobilized on the activated cotton surfaces (LF-cotton) and when the LF-cotton was swabbed on the contaminated chicken meat surfaces, it captured the pathogenic bacteria of interest. In parallel, the black magnetic nanoparticles and corresponding coloured polymeric nanobeads conjugated with their specific antibodies (Ab-D) for each foodborne bacteria were used for colour development. The LF-cotton captured bacteria were incubated with the coloured Ab-D beads. The

colour of the cotton surface indicated the presence of the corresponding bacteria and the detection of the target bacteria was further confirmed by LAMP.

4.4.1. *Salmonella typhimurium*

Lactoferrin linked cotton was swabbed on the St spiked chicken meat, forming LF-cotton-St complexes along with all other bacteria and viruses **(Levay** & **Viljoen,** 1995). The LF-cotton-St complexes were further incubated with the black magnetic beads linked with *S. entirica serovar typhimurium* specific antibody to form a sandwich complex (LF-cotton-St-mAb-D) via antigenantibody interaction, resulting in a colour change of the cotton surface. The free magnetic beads were separated by passing the cotton swab close to a permanent magnetic sheet. This is an advantage over polymer nanobeads as there is no need for washing steps. To determine the lower detection limit of the St bacterial cells, LF-cotton was swabbed over the surface of chicken meat spiked with different concentrations ranging between 10 and 10^8 CFU/ml, followed by the formation of sandwich complexes. The intensity of the black colour on the cotton surface increased with the increasing concentration of bacterial cells as shown in the **Fig.** 4.1.

When LF-cotton was swabbed over a high concentration of bacteria, more cells were captured by the abundant binding sites on the cell surface, thereby forming numerous magnetic bead sandwich complexes. The intense black cotton surface is explained by the formation of more *S. entirica serovar typhimurium* sandwich complexes on the cotton surface at higher cell concentrations (Saleh *et al.,* Unpublished Results). As

shown in **Fig.** 4.1, the visual lower detection limit of the *S. entirica serovar typhimurium* on the chicken surface was as low as 100 CFU/ml. The detection limit of this assay was significantly lower compared to other sensors (**Ahmed** *et al.,* 2014; **Ligler** *et al.,* 2007; **Velusamy** *et al.,* 2010; **Zhao** *et al.,* 2014) developed for the detection of *S. typhimurium*. Recently, a new method Using the immunomagnetic nanospheres and immunofluorescent methods for detection of S. entirica serovar typhimurium was reported, with sensitivity of 10 CFU/ml (**fronczek et al. 2013** and **wen et al. 2013**) which are comparable to the detection limit achieved by this assay. A fluorescently labelled aptamer on a graphene oxide-based biosensor can sense as low as 100 CFU/ml. (Duan et al. 2014). The assay developed in this study is a simple technique in which the pathogenic bacteria on the contaminated surfaces of meat processing plants can be easily identified from the colour of the cotton surface by the naked eye as shown in the **Fig.** 4.1.

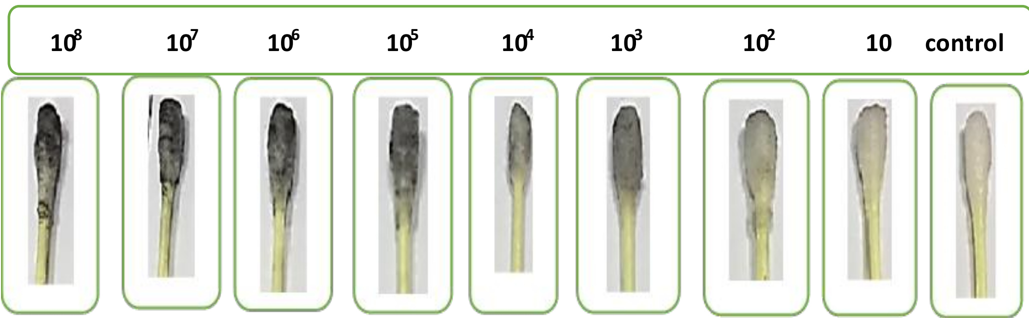


Fig. 4.1: Screening *Salmonella typhimurium* using a cotton swab assay: Lactoferrin immobilized cotton swab and the black magnetic beads captured *S. entirica serovar typhimurium* target cells from chicken meat by sandwich binding. More intense black sandwich complexes were formed with the increasing concentration of bacterial cells. The visual detection limit for the samples from chicken meat is 10^2 CFU/ml.

4.4.2. *Salmonella enteritidis*

A *Salmonella enteritidis* specific antibody was conjugated to blue coloured polymeric nanobeads for the detection of this organism. A LF-Se-D sandwich complex formed when the LF-cotton was swabbed over the contaminated surface, followed by the treatment with antibody linked blue beads. The detection limit was determined by treating the LF with variable S. entirica serovar enteritidis concentrations. The colour intensity of the cotton surface increased with increasing concentration of the bacterial cells used in the range from 10-10^8 CFU/ml (see **Fig.** 4.2). When the cotton-LF was treated with a high concentration of cells, more cells were captured, thereby more beads bound in the second step, explaining the intense blue colour of the cotton surface. The lower detection limit of *S. entirica serovar enteritidis* on the chicken surface was close to 10 CFU/ml (see **Fig.** 4.2). **Song** *et al.* (2014) developed a FRET based biosensor for the detection of *S. enteritis,* with a lower detection limit of ranging from 100 to 1000 CFU/ml. In this study, the quenched fluorophore labelled *S. enteritis* specific aptamer on the graphene oxide was switched on in the presence of *S. enteritis,* with a detection limit of 40 CFU/ml (**Wu** *et al.,* 2014). The new device developed in this study with a low detection limit has the potential for on-site applications.

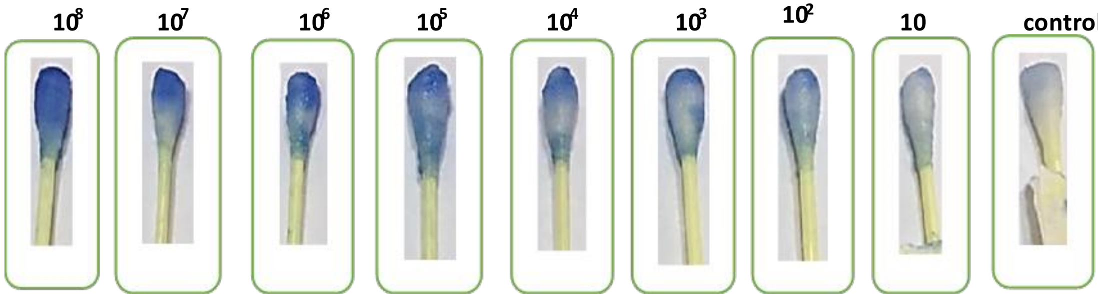


Fig. 4.2: Screening *Salmonella enteritidis* using a lactoferrin immobilized cotton swab: the lactoferrin immobilized cotton swab and the blue magnetic beads captured *S. entirica serovar enteritidis* target cells from chicken meat by sandwich binding. More intense blue sandwich complexes were formed with the increasing concentration of bacterial cells. The visual detection limit for the samples from chicken meat is 10^1 CFU/ml.

4.4.3. *Staphylococcus aureus*

Orange polymeric beads immobilized with *S. aureus* specific antibodies were used for the detection of *S. aureus.* LF captured Sa bacterial cells from chicken meat, forming cotton-LF-Sa-D complexes, which were further treated with colour developing orange polymeric nanobeads linked with Sa specific antibody. The detection limit of the assay was determined by treating the LF with variable concentrations of Sa cells. After sandwich complex formation, the intensity of the orange colour on the cotton surface was proportional to the concentration of cells, due to the availability of more binding sites to bind Sa specific antibody linked beads. The visible detection limit of this assay was 10^3 CFU/ml as shown in the **Fig.** 4.3. An aptamer-based biosensor was reported for the detection of *S. aureus* on skin using single-walled carbon nanotubes (SWCNTs) as an ion-toelectron potentiometric transducer (**Zelada-Guillén** *et al.,* 2012), with a high detection limit of 10^3 CFU/ml. Highly

sensitive methods with less than 10 CFU/ml have been reviewed recently (**Ahmed** *et al.,* 2014; **Zhao** *et al.,* 2014). The detection limit of a dropsens screen printed electrode based immunosensor achieved a detection limit as low as 1 CFU/ml (**Esteban-Fernández de Ávila** *et al.,* 2012**)**. Multiple pathogen (including Sa) detection methods were also reported using the fluorescence sensor arrays (**Mungkarndee** *et al.,* 2015). Nonetheless, our method is simple, easy to perform and is a colorimetric method which can be seen with the naked eye.

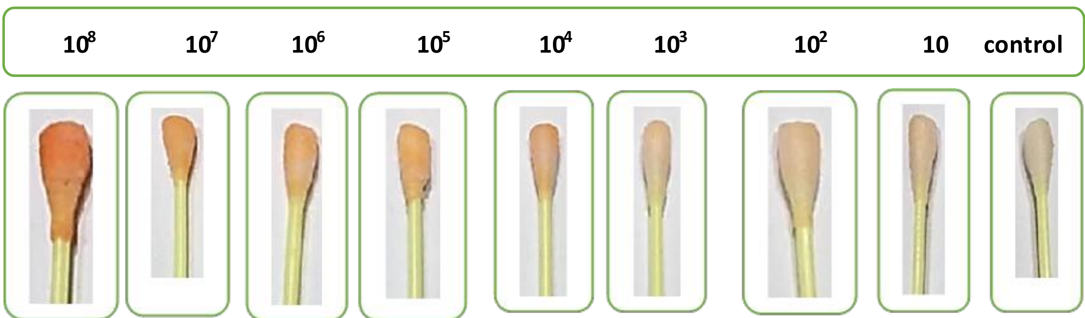


Fig. 4.3: Screening of *Staphylococcus aureus* using a lactoferrin immobilized cotton swab: The lactoferrin immobilized cotton swab and the orange magnetic beads captured the *S. aureus* target cells from chicken meat by sandwich binding. More intense orange sandwich complexes were formed with the increasing concentration of bacterial cells. The visual detection limit for the samples from chicken meat was 10^3 CFU/ml.

4.4.4. *Campylobacter jejuni*

Green polymer nanobeads linked with *Campylobacter jejuni* binding monoclonal antibody were used for the development of a screening assay for *C. jejuni*. The increasing intensity of green sandwich (LF-Cj-D) complexes were formed on the cotton surface on treatment with serial dilutions of cells, with the intensity of the green colour proportional to the increasing numbers of cells in the samples. This assay sensed as low

as 100 CFU/ml of Cj from chicken meat surfaces as shown in the **Fig.** 4.4. **Viswanathan** *et al.* (2012) developed electrochemical immunosensors for the detection of multiple pathogens in food using a nanocrystal bio-conjugate and multi walled carbon nanotube screen printed electrodes, which could detect 400 CFU/ml of *C. jejuni* in milk samples. A nanoparticle enhanced QCM based immunosensor detected as low as 150 CFU/ml of *C. jejuni*, which is comparable to our assay detection limit (**Masdor** *et al.,* 2016). However, the newly developed system is low cost, easy to use and has the capacity for on-site detection of *C. jejuni*. Correlation between colour intensity and concentration of cells in samples which is shown in fig (4.7) refers to the possibility of using this assay not only in qualitative analysis but also in semi quantitative.

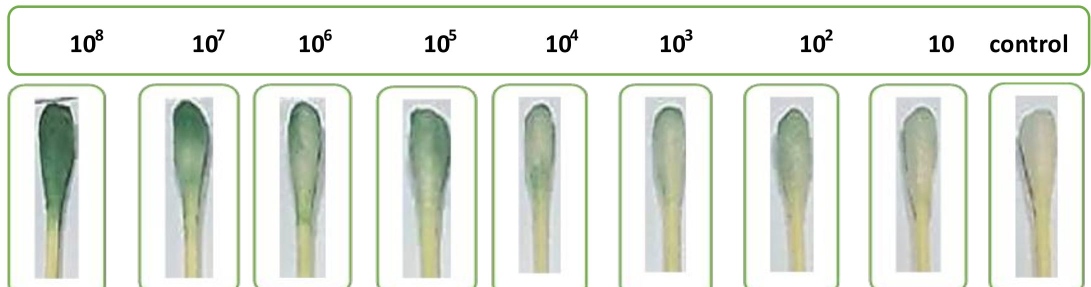


Fig. 4.4. Screening *of Campylobacter jejuni* using lactoferrin immobilized cotton swab and green magnetic beads. The *C. jejuni* target cells from chicken meat were captured by sandwich binding. More intense green sandwich complexes were formed with the increasing concentration of bacterial cells. The visual detection limit for the samples from chicken meat is 10^2 CFU/ml.

4.5. Specificity tests

The success of sensor performance mainly depends on the specificity of the method, which in this assay is due to the selectivity of the detection antibodies linked with coloured beads. Therefore, the cross reactivity of the antibodies used in the sensor development were tested using four

closely related bacterial strains, *S. enteritis, S. aureus, E. coli* and *C. jejuni*. Four cotton-LF swabs were treated individually with St, Se, Sa and Cj and each cotton swab was further incubated with anti-*S. entirica serovar typhimurium* antibody linked black magnetic beads. Only the cotton treated with *S. entirica serovar typhimurium* developed a black colour as shown in **Fig.** 4.5, thereby confirming the specificity of the assay. Similarly, the specificity of Se, Sa and Cj sensors were confirmed as shown in the **Fig.** 4.5.

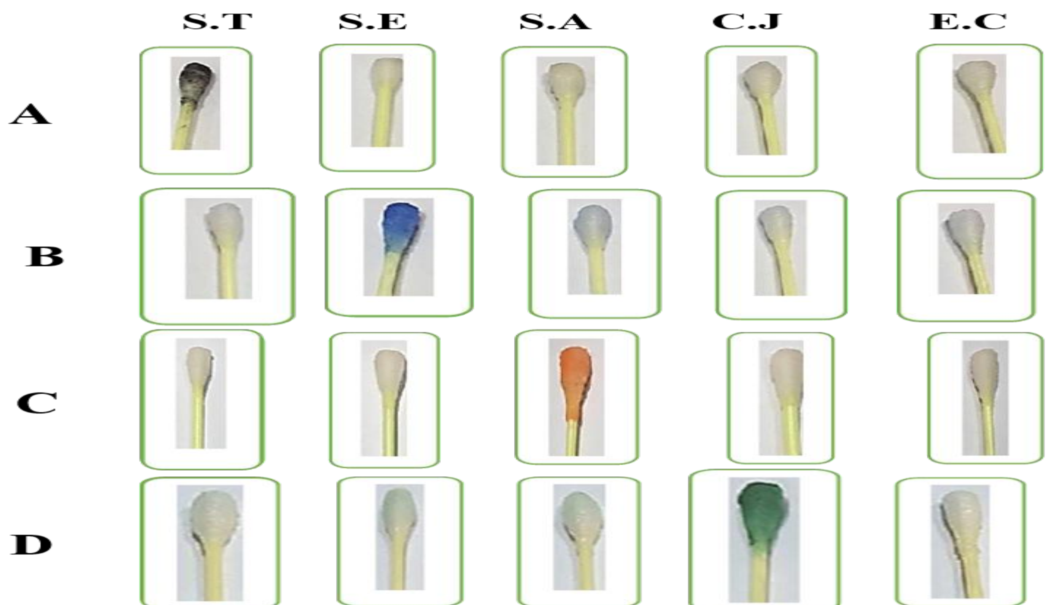


Fig. 4.5: Evaluation of Specificity for the sensors: the chicken meat surfaces with five different pathogenic bacteria, St, Se, Sa, Cj and *E. coli,* were swabbed with five lactoferrin linked cotton swabs. Each cotton swab was treated with a St specific antibody linked black magnetic beads and only St treated cotton turned black (A). Similarly, Se treated cotton surface changed to blue (B) via sandwich complexed with blue beads coupled with anti-Se antibody. Sa treated cotton surfaces changed to orange (C) via sandwich complexed with orange beads coupled with anti-Sa antibody and Cj treated cotton surface changed to green (D) by sandwich complexed with orange beads coupled with anti-Cj antibody.

4.6. LAMP

The specificity of the developed assay was also assessed by LAMP. Artificially contaminated chicken surfaces with different concentrations of bacterial pathogens were swabbed with lactoferrin linked cotton Q-tips and immersed in Tris-HCl buffer (10 mM , P^H 7.4). The Q-tips were incubated in the buffer for 5 min at 95°C, then transferred to commercially available LAMP master mix (including the sample, primers, enzyme, dNTPs) and 1 µl of HNB dye was added, followed by 60 min incubation at 65°C. The interaction of the amplification products which include magnesium with HNB resulted in a colour change of the dye from violet to sky blue, indicating the presence of the target gene of the pathogenic bacteria as shown in **Fig.** 4.6.

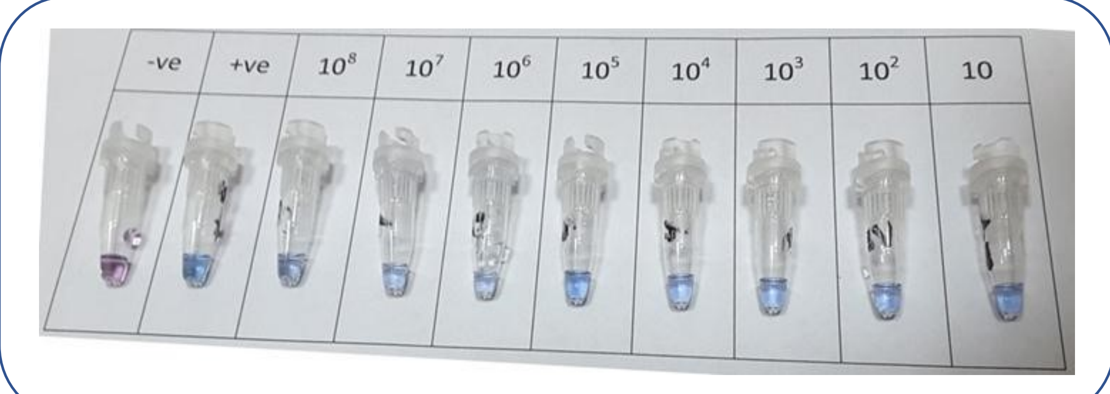


Fig. 4.6. LAMP confirmation test: The cotton-lactoferrin capture of *C. jejuni* from the chicken meat was confirmed by the amplification of its specific gene by LAMP. The change in the colour of the HMB dye from purple to blue indicated amplification of the target gene, while there was no amplification in the purple coloured negative control.

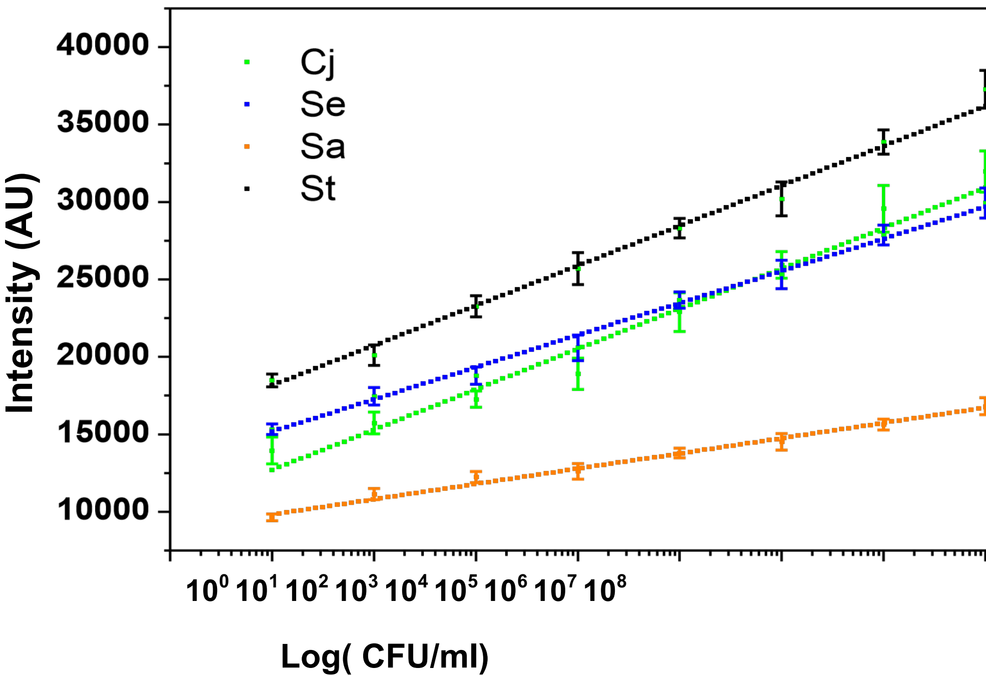


Fig. 4.4: calibration curves: indicating the Correlation of colour intensity of the cotton swap with bacterial concentration.

4.7. Innovative use for lactoferrin

Because of the unique characteristics of lactoferrin as an iron binding protein, most previous studies have concentrated on using lactoferrin – extracted from various sources – as an antibacterial agent. In (Rasima et al. 2014), lactoferrin, isolated from colostrum of cows was used to reduce the formation of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms. This ability to reduce biofilm formation was explained by its ability to scavenge free ions of iron, so causing an imbalance in the environment around the bacteria. In another study (Seyyed Mohsen Sohrabi et al. 2014), lactoferrin, isolated from camel milk, was used as an antimicrobial agent against *Staphylococcus aureus, Escherichia coli* and *Candida albicans*. In addition to the use of lactoferrin as an antimicrobial agent, some other studies have used the ability of lactoferrin to bind different

bacterial surfaces to develop or to improve label-based detection methods. In (Melin, J et al. 2008), labeled lactoferrin and lactoferricin have been used instead of antibodies as binding reagents in an antibody chip. It was found that the fluorescence of lactoferrin binding was at levels similar to those demonstrated in the results obtained with boro-dipyrromethene boron fluoride (BODIPY), a lipid probe with excitation/emission at 530–550 nm, and to the anti-Salmonella antibody (Cimaglia, et al. 2014). In the present study, lactoferrin was used in a more effective way. It was used, free of labeling, to collect bacterial cells from different surfaces using cotton buds. Then a sandwich assay was undertaken using specific antibodies which were previously immobilized on dyed nano beads. The results obtained in this study showed a very good sensitivity and selectivity of the assay with a very low detection limit, reaching 10 to 100 CFU/ml. These results are also comparable with those reported in previous studies which used antibodies and a recognition element. In (Yi Wang et al. 2012), E. coli was detected at a detection limit of 50 CFU/ml. using SPR based on the spectroscopy of a grating-coupled long-range surface combined with magnetic nanoparticles. In another study, Campylobacter jejuni was detected at a limit of detection of $8x10^6$ (Masdor et al. 2017), and in a previous study by the same author, campylobacter was detected at a detection limit of $1x10^5$ (Noor a zlina Masdor et al. 2016).

4.8. Conclusions

The newly developed immune sensor for the detection of pathogenic bacteria on chicken meat is simple, rapid, low cost, reliable and portable

for on-site applications. The assay used lactoferrin as a recognition receptor for all bacterial pathogens tested, followed by the formation of sandwich complex between each pathogen and its specific antibodies. The bacterial strains on the contaminated food were identified by the colour of the cotton surface after the sandwich complex formation, as each colour was specific for a single type of bacteria. The assay was used to identify the presence of *S. typhimurium, S. enteritidis*, *S. aureus* and *C. jejuni* by the change in the colour of the cotton surfaces, and were further validated by LAMP. The colour intensity of the cotton surface was directly proportional to the concentration of pathogenic bacteria present on the chicken meat, indicating that the method can be applied, not only for qualitative, but also for semi-quantitative determination. The lower detection limits of the bacterial cells were in the range of 10 to 100 CFU/ml, which is comparable with recently reported values. However, this assay does not require sophisticated instruments and the pathogens can be identified by naked eye. The specificity of the assay is promising when tested with closely associated bacteria. As it is a simple, specific and visual colorimetric detection method, this biosensor has the potential for application in the field for the rapid screening of bacterial contamination in food products (chicken, ready to eat foods, ground meat) and other biomedical, environmental and security applications. Work is ongoing in our lab to integrate this assay on a chip for point-of-care applications.

Chapter 5: Development of a simple, fast and cost effective nano-based immunoassay method for detecting *Norovirus* in food samples

5.1. Abstract

This study presents a quick, cheap, and easy technique for the detection of *Norovirus* in different food samples, including cucumber, lettuce and chicken. This sandwich immunoassay method depends on nanotechnology for the detection step and lactoferrin (immobilized on activated cotton swabs) was used as a general capturing reagent to bind virions from the sample surface. The cotton swabs were then immersed in a gold nanoparticle solution, which had been previously immobilized with a specific antibody. Positive samples retained the red colour of gold nanoparticles on the surface of cotton swabs, even after washing, while the negative samples easily lost their colour through washing. The results showed that the assay had very good sensitivity and selectivity, with a LOD of 10 PFU/ml for all tested samples. In light of the difficulty, complexity and high cost of the methods recently used for detecting viruses in food samples, this method presents a promising reliable technique which can be used for the rapid detection of *Norovirus* in food samples with an acceptable accuracy.

5.2. Introduction

Recently, viruses have been reported as one of the major causes of foodborne illnesses. In USA, viruses are responsible for 66.6 % of food related diseases compared with 9.7 % caused by *Salmonella* and 14.2 % caused by *Campylobacter* (**Mead** *et al.,* 1999; **Vasickova** *et al.,* 2005). *Norovirus* is one of the most wellknown viruses causing foodborne diseases, being responsible for 45% recreational waterborne outbreaks, followed by *Adenovirus* which is responsible for 24% (**Sinclair** *et al.,* 2009;

LaRosa *et al.,* 2012). *Norovirus* was first recognized in 1968 in Ohio, United States during the outbreak of a winter vomiting disease (**Adler** & **Zickl**, 1969). As reported by **Zheng** *et al.* (2010), *Norovirus* belongs to a genetically diverse group of non-enveloped, single stranded RNA viruses of the family called *Caliciviridae*. This group is currently subdivided into five subgroups, *Norovirus*, *Sapovirus, Lagovirus, Vesivirus,* and *Nebovirus*, with one or more species recognized in each genus. *Norovirus*es are responsible for acute gastroenteritis outbreaks around the world. In the USA for example, there are 5.5 million annually who suffer from foodborne *Norovirus* diseases (**Hall** *et al.,* 2012), and in the UK, there are 600,000 cases of *Norovirus* infections (**Wheeler** *et al.,* 2005). *Norovirus*es are known to be resistant to many disinfectants, so they remain infectious for about two weeks on surfaces and for more than two months in water (**Seitz** *et al.,* 2011; **Park** *et al.,* 2011). *Norovirus* can be spread in different ways, including direct transmission from one person to another via faeces or ingestion. Also, it can be transmitted indirectly through contaminated surfaces, water or food (**Hall** *et al.,* 2012). What makes *Norovirus* very dangerous and highly contagious, is that a very small dose, less than 10^2 copies/ml, can cause an infection in humans and it is highly resistant to heat up to 60°C and acids, such as the chlorine in tap water, up to 6.25 mg/l (**Neethirajan** *et al.,* 2017).

Although there are many detection methods available for viruses in food, including *Norovirus* like ELISA, electrophoresis and chromatography, the molecular method remains the most commonly applied technique due to its high sensitivity and selectivity. The molecular detection of *Norovirus* in food matrices by RT-PCR is a widely used method in research

laboratories, comprising three main steps: virus extraction, purification of RNA, and the molecular detection of RNA. Extraction methods used to isolate virus molecules from food matrix vary according to the biological composition of each matrix (**Baert** *et al.*, 2008). There are three main categories of food matrices; the first one is waterbased foods including carbohydrates like fruits and vegetables, the second is protein-based including fats (mainly ready to eat products and chicken meat) and third category is shellfish, which contains viruses in their digestive systems. Virus extraction includes the elution of viral particles, the direct extraction of viral RNA from the food matrix and the extraction of virus by proteinase K treatment. However, the sensitivity and the selectivity of this procedure is complex, time consuming and costly. Recently, we developed a new approach for detecting *Norovirus* in vegetables and chicken meat by an immunoassay method which is simple, fast and cost effective. This sandwich immunoassay technique uses cotton buds immobilized with lactoferrin as a general binding reagent and gold nanoparticles immobilized with a specific antibody as a specific binding reagent.

5.3. Screening procedure

The screening procedure consists of two steps. The first step is virus capture and the second step is the sandwich formation with the secondary antibody and colour development. In this study, lactoferrin (LF) was used as a universal recognition receptor, as it can bind any virus in the sample. Lactoferrin was conjugated to the cotton swab and the gold nanoparticles conjugated to the secondary detection antibodies (D-

mAb) used for the colour development. In the first step, the cotton immobilized lactoferrin was swabbed over the contaminated sample surfaces to capture the *Norovirus.* The cotton immobilized lactoferrin-virus complexes were then washed twice with PBS to remove the free virus molecules.

In the second step (detection step), the virus was sandwiched between the cotton immobilized lactoferrin and secondary detection antibody conjugated with coloured gold nanoparticles. The cotton swab lactoferrin-virus (cottonLF-virus) complexes were immersed in solution of gold nanoparticles linked with the secondary antibody in PBS for 2 min. The LF-virus-secondary antibody (LFvirus-mAb-D) sandwich complex was washed with PBS to remove the unbound beads. The colour of the cotton swab indicates the specific viral stain present on the contaminated surfaces.

5.4. Results and Discussion

In this study, a simple sandwich immune-based colorimetric method was evaluated for the detection of *Norovirus* in two types of vegetable samples in addition to chicken meat as common potential foodstuffs, lettuce, cucumber and chicken meat. They are more likely to be contaminated with the virus during harvesting, transport as well as storage processes.

5.4.1. Principle and efficiency of the assay

Samples (chicken, lettuce and cucumber) spiked with *Norovirus* were swabbed using lactoferrin immobilized cotton buds, to allow the

formation of LF-cottonvirus complexes along with viral particless or any other bio-macromolecules, cations or anions **(Levay** & **Viljoen,** 1995) through the interaction between lactoferrin molecules and the biological lipopolysaccharide (LPS). Then, the cotton buds were incubated with red coloured gold nanoparticles which were previously immobilized with a secondary antibody specific to *Norovirus.* The combination of the LF-virus complex and gold nanoparticles immobilized with secondary antibodies leads to change in the colour of the cotton swap to red. The linearity and sensitivity (which can be expressed as a limit of detection) of the assay were evaluated in the range of 10^5, 10^4, 10^3,$10^2$and 10 PFU/ml. This concentration series was used to spike samples and then subjected to sandwich immunoassay process as discussed previously.

The results showed a very high sensitivity and good linear regression between the virus concentration and colour intensity in all samples, which can be explained by the high number of nanoparticles collected in turn. The limit detection for this assay for the detection of *Norovirus* in both lettuce and cucumber samples was reported as 10 PFU/ml as shown in **Fig.** 6.1 and **Fig.** 6.2. The linearity and the sensitivity of the assay in chicken samples was not different from that in vegetables samples, i.e. the same limit of detection of 10 PFU/ml as shown in the **Fig.** 6.3. The limit of detection was similar in the cucumber and lettuce samples, but the colour variation between different concentrations was not as clear as shown in **Fig.** 6.2. This is because of the low intensity of the colour in general and may be related to the physical characteristics of the cucumber surface which is smoother than lettuce, so may adhere less virus molecules than lettuce samples. The correlation of the colour

intensity of a cotton swap with concentration of virus molecules captured was confirmed by establishing a relationship between integrated intensity values calculated by Photoshop software and concentration as shown in **Fig.** 6.4.

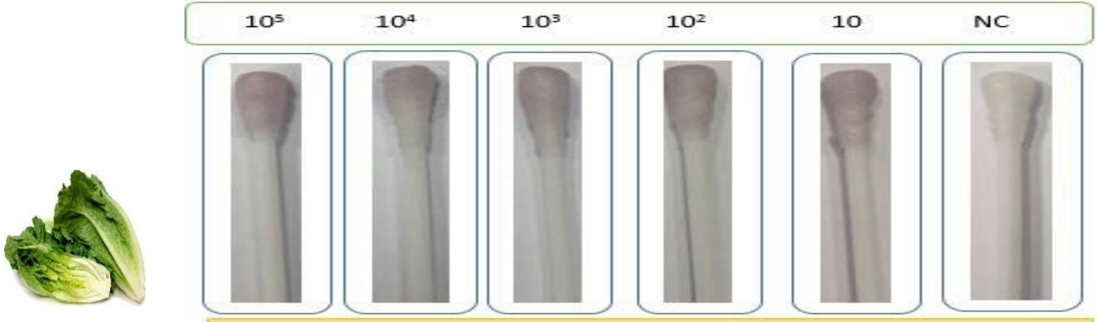


Fig. 6.1: Gradient of colour intensity with the concentration of *Norovirus* spiked on lettuce Within the range (10^5 to 10) spiked in lettuce samples.

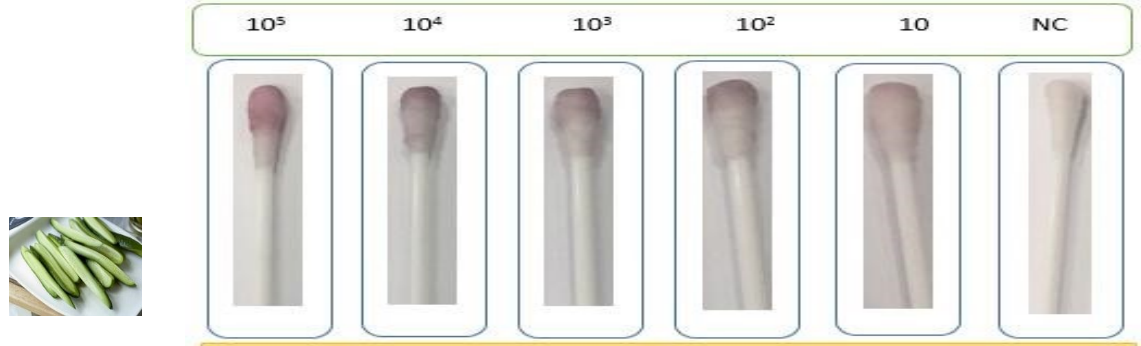


Fig. 6.2: Different concentrations of *Norovirus* within the range (10^5 to 10) spiked in cucumber samples.

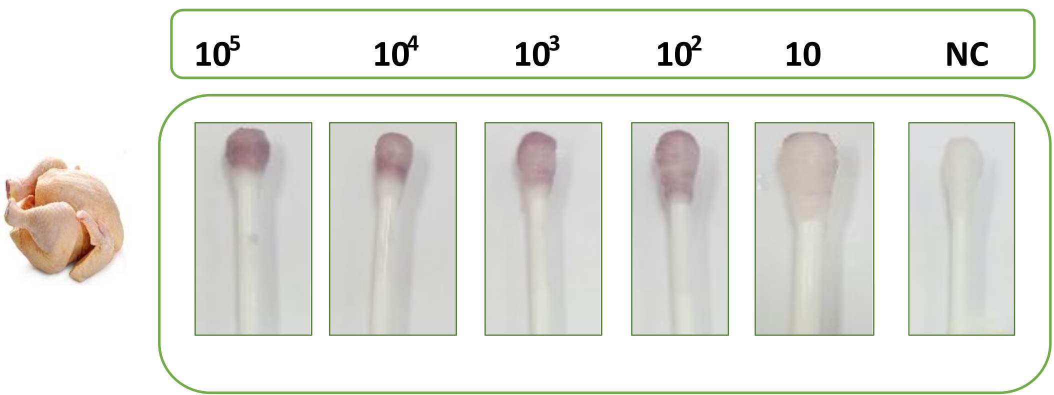


Fig. 6.3: Different concentrations of *Norovirus* spiked chicken within the range (10^5 to 10).

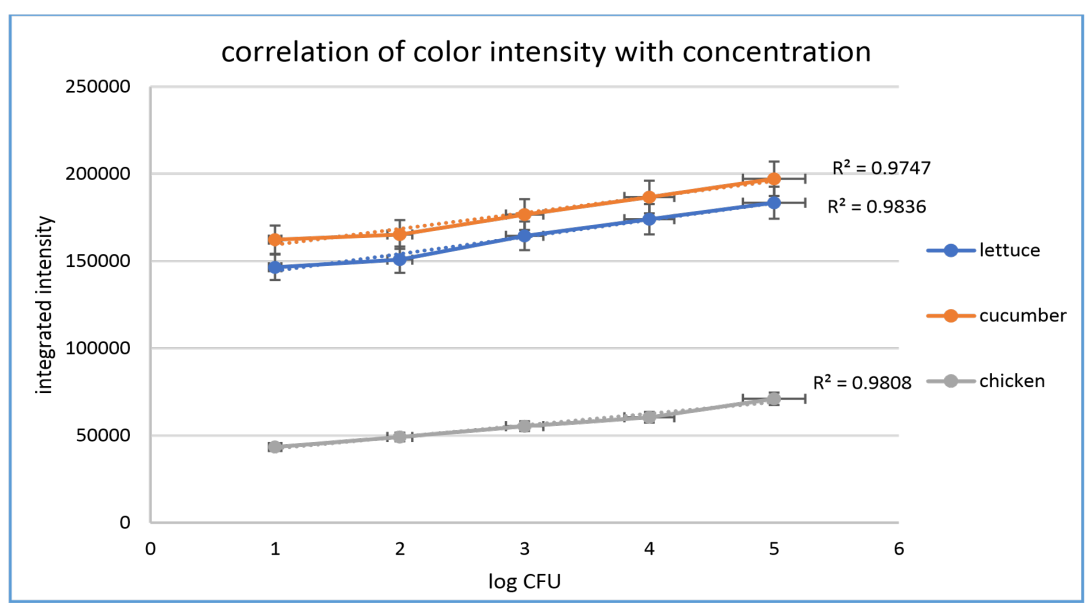


Fig. 6.4. Calibration plots, indicating the linear correlation between colour intensities and Bacterial concentrations in three sample matrices

5.4.2. Comparison between the developed assay and other techniques in terms of diversity, simplicity, complexity and cost

The newly developed technique resolves some of the many challenges of known techniques for the detection of *Norovirus.* For example, electron microscopy (EM) technique was the first tool used for detecting *Norovirus,* but requires a high viral load in the sample for analysis. However, the sensitivity can be improved by using immune-EM, as well as other techniques like traditional immunoassay, ELISA and RT-PCR, which are nowadays the most widely used molecular techniques for detecting *Norovirus*. The major disadvantage of EM and ELISA is the low sensitivity, which falls between 10^6 and 10^4 virus particles per gram of sample. Furthermore, the main limitation of using EM and RT-PCR is the fact that *Norovirus*es are genetically highly diverse, and this complicates the design of protocols to detect multiple strain variants. In addition,

inhibitors (humics, complex polysaccharides, microorganism debris, metal ions and nucleases) in samples may inhibit the amplification process during RT-PCR. The following table explains the main differences between the most widely used techniques for the detection of *Norovirus* in different samples and the advantages of the developed assay over the other techniques. The comparison of the study results with other studies, shows that the developed assay has a very high sensitivity in terms of the limit of detection. The limit of detection in a previous study using phage nanoparticle reporters in a lateral flow assay was 10^7 virus particles per ml (**Hagström** *et al.,* 2015). In another study, the limit of detection using three-dimensional paper-based slip device for one-step pointof-care testing was 9.5×10^4 copies per ml for human *Norovirus* (**Han** *et al.,* 2016).

Table 6.1: Comparing the new assay with some other techniques in terms of diversity, simplicity, complexity and cost

Technique	Sensitivity	Process simplicity/ complexity	Limitation	Cost
Microscopy (EM)	**Low**	**Simple**	• Need high viral load in the sample	High
Traditional immunoassay (ELISA)	**Low**	**Complex**	• High diversity complicates the protocol • Need high viral load in the sample	High

Molecular (RT-PCR)	**Good**	**Complex**	• High diversity complicates the protocol	High
Recent study (Nano-based immunoassay)	**Good**	**Simple**	• High diversity complicates the protocol	Low

5.5. Conclusion

This study developed a simple, fast, easy, and cost-effective method for detecting *Norovirus* in food samples. The technique depends on a sandwich immunoassay technique using lactoferrin immobilized on cotton buds as a general binding element, which can bind many biological molecules including bacteria, viruses and DNA. The detection of virus molecules was achieved by gold nanoparticles which had been previously immobilized with specific antibody; the colour change of the cotton buds after immersion in the nanoparticle solution indicates positive samples. The results indicate that the method is highly reliable regarding the detection of *Norovirus* in various food samples, particularly, lettuce, cucumber and chicken. The correlation between colour intensity and concentration of the analyte in solution indicates that the assay can be used for quantification. Also, the limit of detection was 10 PFU/ml in the samples tested, so this assay technique could be suitable for many applications in the food industry, food safety and biodefence.

Chapter 6: Sensitive graphene oxide-based fluorescence biosensor for the detection of *Salmonella enteritis* using a truncated DNA aptamer

6.1. Abstract

This fluorescence-based study mapped the highest affinity truncated aptamer from the full-length sequence and integrated it in a graphene oxide platform for the detection of *Salmonella enteritidis*. Molecular beacons as well as displacement assay design were used to identify the best truncated sequence. In the fluorescence displacement assay, the truncated aptamer was hybridized with fluorescein and quencher labelled complementary sequences to form a fluorescence-quencher pair. In the presence of *S. enteritidis,* the aptamer dissociated from the complementary labelled oligonucleotides and thus, the fluorescein-quencher pair became physically separated, leading to an increase in the fluorescence intensity. One of the truncated aptamers showed a two-fold (3.2 nM) increase in the dissociation constant compared with its full-length aptamer (6.3 nM). Then, the selected truncated aptamer was used to develop a simple fluorescence-based graphene oxide sensing platform. To fabricate the sensing platform, the fluorescein labelled aptamer was adsorbed on the GO surface by π-π stacking interaction, leading to quenching of the fluorescence intensity. However, in the presence of target molecule, the labelled aptamers were released from the GO surface forming a stable complex with the target bacteria cells. The detection limit of the aptasensors fabricated using the truncated aptamer was lower than the full-length aptamer. Moreover, the aptasensors did not show significant cross reactivity with other related bacteria such as *S. typhimurium*, *S. aureus* and *E. coli*. The fluorescence/graphene oxide aptasensors have also shown good recovery for the detection of *S. entirica serovar enteritidis* from spiked

milk samples. Thus, the truncated aptamer/graphene oxide platform is a potential candidate method for the detection of *S. entirica serovar enteritidis* for biomedical and environmental applications.

6.2. Introduction

Aptamers are considered as chemical antibodies which can be selected for various analyses for clinical, food and environmental applications (**Tombelli** *et al.*, 2005; **Song** *et al.,* 2012; **Sun** & **Zu**, 2015). Aptamers are short synthetic DNA or RNA sequences which can specifically capture their analytes with high affinity (K_ds in the nanomolar to picomolar range). Specific aptamers for proteins (**Eissa** & **Zourob,** 2016), metal ions (**Wrzesinski** & **Jóźwiakowski,** 2008), bacteria (**Duan** *et al.,* 2014), viruses (**Gonzalez** *et al.,* 2016) and small molecules (**Elshafey** *et al.,* 2015; **Ruscito** & **DeRosa**, 2016; **Alhadrami** *et al.*, 2017) have been selected using *in vitro* selection protocols. Aptamers have been widely exploited in biosensor development, showing many advantages over antibodies such as their high stability, low cost, ease of chemical synthesis and modification. The typical aptamer usually consists of 40 to 100 nucleotides that under favourable conditions fold into secondary or tertiary structures, which can bind to a target molecule to form stable target-aptamer complex.

Fluorescence-based aptasensors are usually designed to undergo structure switching or conformational changes on binding with the target (**Miso** *et al.,* 2016; **Mallikaratchy,** 2017). However, it is not easy to predict the occurrence of the conformation change within the full-length aptamer. Moreover, slicing the non-binding region of the aptamer favours the formation of a stronger complex with the target (**Le** *et al.,* 2014; **Zheng** *et al.*, 2015; **MacDonald** *et al.,* 2016). For instance, the

affinity of the truncated aptamer of vascular endothelial growth factor ($VEGF_{165}$) increases by 200 fold compared to the wild type aptamer (**Kaur** & **Yung**, 2012). Recently, our group reported a 17-fold increment in the affinity of the truncated anti-progesterone aptamer compared to the parental aptamer (**Alhadrami** *et al.,* 2017). Therefore, mapping the binding site within the aptamer sequence represents an important step for increasing the affinity and the conformation change of the aptamer. Graphene oxide (GO) is a two dimensional carbon nanomaterial with unique properties due to its electronic configuration (**Zhu** *et al.,* 2010), large surface area and high dispersion capability in water (**Chung** *et al.,* 2013). These unique properties make GO an ideal material for the development of environmentally-friendly and low cost biosensor platforms (**Jung** *et al.,* 2010). GO is a good energy acceptor, therefore, it has been used as a sensing platform for the detection of different biomolecules using on/off fluorescence assays (**Chang** *et al.*, 2010; **He** *et al.,* 2011; **Cai** *et al.,* 2014; **Duan** *et al.,* 2014). Graphene oxide-based aptasensors were developed for the detection of thrombin and ATP, in which the fluorescence of the fluorescent aptamer was quenched by GO and then restored in the presence of the target analyte.

In this work, different fluorescence assays were used to select the highest affinity truncated aptamer for *S. entirica serovar enteritidis* for the development of an aptamer-based fluorescence GO sensor for the sensitive detection of S. *enteritidis*.

6.3. Results and Discussion

Generally, the DNA library sequences used for the SELEX screen should be of a certain length (between 40-100 nucleotides) to increase its diversity and maximize the probability of selecting high affinity aptamers for the target. However, not all the nucleotides in the aptamer are involved in the critical binding with the target molecules. In most cases, the parts of the aptamer sequence which form the stem-loop structure, G-quartet, bulges and/or pseudoknots are involved in the direct binding with the target molecules (**Jayasena**, 1999; **Cowperthwaite** & **Ellington**, 2008; **Gao** *et al.,* 2016). Some additional nucleotides may be essential for supporting the contact between the target molecule and the aptamer. However, the rest of the aptamer sequence which is not involved in the binding can destabilize the aptamer-target complexes (**Zhou** *et al.,* 2011). Moreover, long aptamers are not usually compatible with biosensors which work based on the confirmatory change. Therefore, post SELEX modification of aptamer is needed for the development of sensitive biosensors (**Maehashi** *et al.,* 2007). Recently, **Kolovskaya** *et al*. (2013) selected high affinity and specific aptamers for *S. entirica serovar enteritidis* using *in vitro* selection from a pool of 2 x 10^{15} DNA sequences. The K_d of the full-length aptamers consisting of 80 nucleotides, including the primer binding sites at 3'and 5' ends, was 80 nM. However, after elimination of 25 nucleotides from the 5' end, including the primer binding site, the remaining sequence consisting of 54 nucleotides showed improved affinity with a k_d of 6.3 nM. The antibacterial activity of the shorter aptamer was increased to 42% compared to the full length

aptamer (9%) (**Kolovskaya** *et al.,* 2013). The aim of this study is to further truncate the sequence to improve the affinity and exploit the shorter sequence in a simple GO fluorescence biosensor for the detection of S. *enteritidis*.

6.3.1. Selection of the highest affinity truncated aptamer

The secondary structure obtained from the mfold software of the full-length aptamer is shown in **Fig.** 5.1. In order to identify the short aptamer sequence that contains the target binding domain, the SE54 (**Kolovskaya** *et al*. 2013) aptamer which was obtained from a previous published study (Kolovskaya et al. 2013) was divided into three different parts. Two stem-loop structures (SE54MB1 and SE54MB2) from both ends and a truncated part from the middle region were investigated in this study. For the stem-loop design, nucleotides were added or modified in the stem region to make the perfect stem. Then, the two ends of the stem were labelled with fluorescein and BHQ1 to form an aptamer beacon. The aptamer beacon, SE54MB1, consisted of 16 nts (3-18) from the 5'region of the full aptamer SE54 and SE54MB2 was a 22mer (27-48) from the 3'region of SE54. Out of the 22 nucleotides of the aptamer SE54MB2, 15 nucleotides represented the constant primer binding site sequences. The sequences used in the study are mentioned in table **5.1**. No significant change in the fluorescence intensity of both SE54MB1 and SE54MB2 after incubation with *S. entirica serovar enteritidis* bacteria was observed as shown in (**Fig 5.2**). These results indicate that both SE54MB1 and SE54MB2 did not undergo a conformation change that could interrupt the fluorescein-quencher pair in the presence of *S. enteritidis*. In other words, the

nucleotides for SE54MB1 and SE54MB2 were not sufficient for target binding and some other nucleotides from the middle part are essential for binding. In fact, it is expected that the constant region of the aptamer (primer binding region) does not contribute to the binding properties of the aptamer or has minimal involvement compared to the overall structure of the aptamer (**Gao** *et al.,* 2016).

After it was established that the two hairpins at both ends of the aptamers did not bind to the target, the sequence taken from the middle (SE54T) was then investigated. Two short complementary sequences of the SE54T aptamer forming the fluorescein and quencher pair were used as reporters for target binding to the aptamer. The reporting complementary sequences were hybridized to SE54T to form a DNA duplex, in such a way for the fluorescein and the quencher to be in close proximity to each other when the duplex was formed. Upon addition of the target molecule, the aptamer bound to the target, changing its conformation, leading to complete or partial displacement of either one or both complementary sequences. These changes led to the physical separation of fluorescein and BHQ1, which could be detected via the increase in the fluorescence as shown in **Fig.** 5.3. A, black, after duplex formation (blue) and the fluorescence recovery after *S. entirica serovar enteritidis* binding (red). When an equal concentration of SE54T and the reporter sequences, SE54C1 and SE54C2, are duplexed, 75% of the fluorescence intensity was quenched compared to the free fluorescein labelled SE54C1, indicating the perfect duplex formation. The decrease in the fluorescence intensity revealed that the fluorescein and the BHQ1

are in close contact to each other as required in the fluorescence displacement sensor. As shown in **Fig.** 5.3 B, the fluorescence intensity increased drastically with the addition of *S. enteritidis*, implying that the fluorescein and the BHQ1 were separated due to either dissociation of one or both complementary sequences. The limit of detection (LOD) of this assay was 30 CFU/ml. These results indicated that SE54T is involved in *S. entirica serovar enteritidis* binding. Furthermore, the truncated part, SE54T, has a stemloop secondary structure as shown in **Fig.** 5.1, which may form the binding pocket for the *S. entirica serovar enteritidis* surface protein. In addition, the specificity of this truncated aptamer was high, as evidenced in specificity tests against *S. typhimurium*, *S. aureus* and *E. coli* (see **Fig.** 5.3 C). Therefore, this truncated part was selected for the GO platform to develop a simple fluorescence-based biosensor.

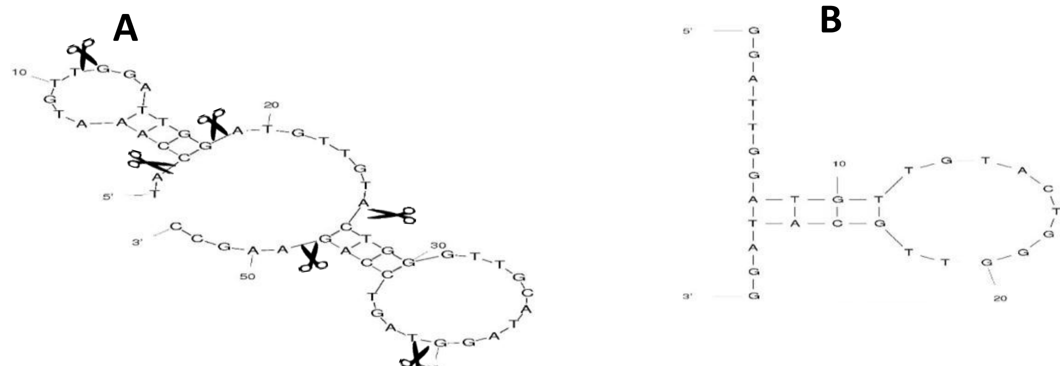


Fig. 5.1: The secondary structure predicted from the full-length aptamer sequence (SE54) and the truncated aptamer SE54T using mfold software.

Table 5.1: The aptamer sequences used in this study.

Name	Sequence from 5'to 3'
SE54F	Flu-TACCAAAATGTT**GGATTGGATGTTGTACTGGGTTGCATAGG**TAGTCCAGAAGCC
SE54T	GGATTGGATGTTGTACTGGGTTGCATAGG
SE54TF	Flu-GGATTGGATGTTGTACTGGGTTGCATAGG
SE54TC1	Flu-ACAACATCCAAT
SE54TC2	ATGCAACCCAGT-BHQ1
SE54MB1	Flu-CGGGTTGCATAGGTAGTCCG- BHQ1
SE54MB2	Flu-ACCAAAATGTTGG-BHQ1

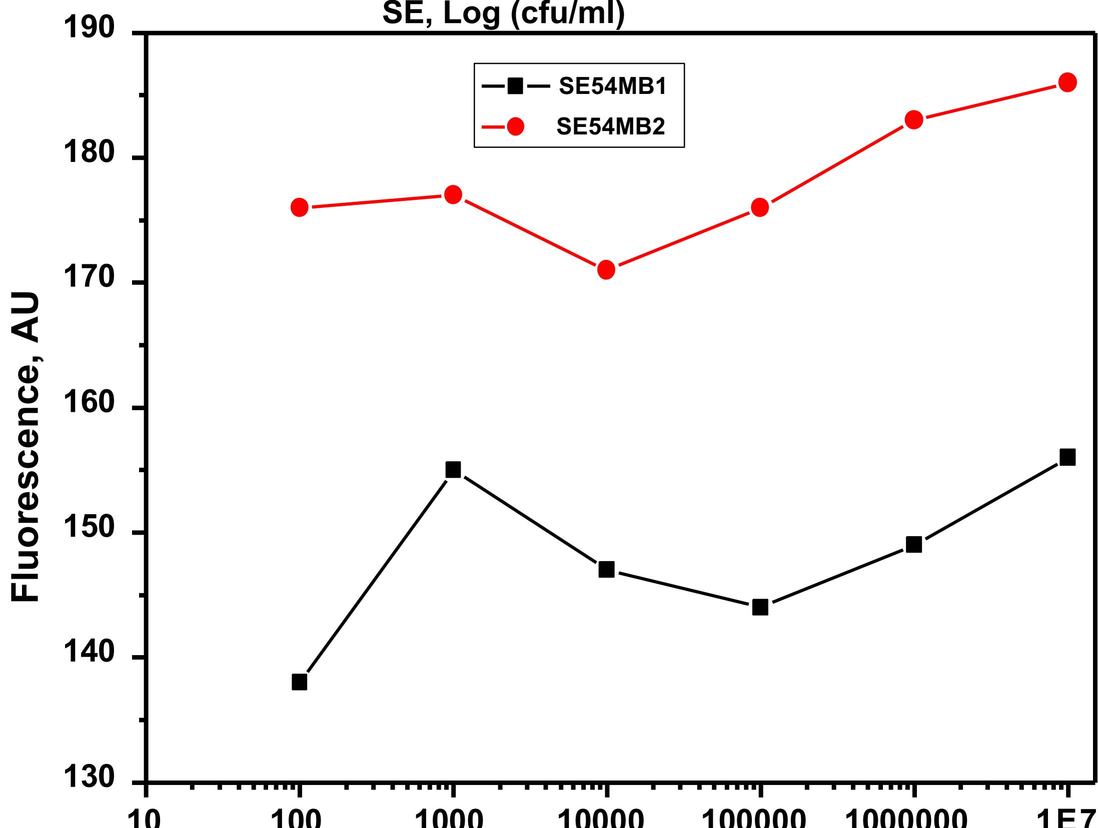


Figure 5.2 : Change in the fluorescence intensity of SE54MB1 and SE54MB2 with increasing concentration of S. *Enteritidis. There is no significant change with increasing concentration of S. Enteritidis, indicating that these* sequences, SE54MB1 and SE54MB2 did not bind to *S. Enteritidis*.

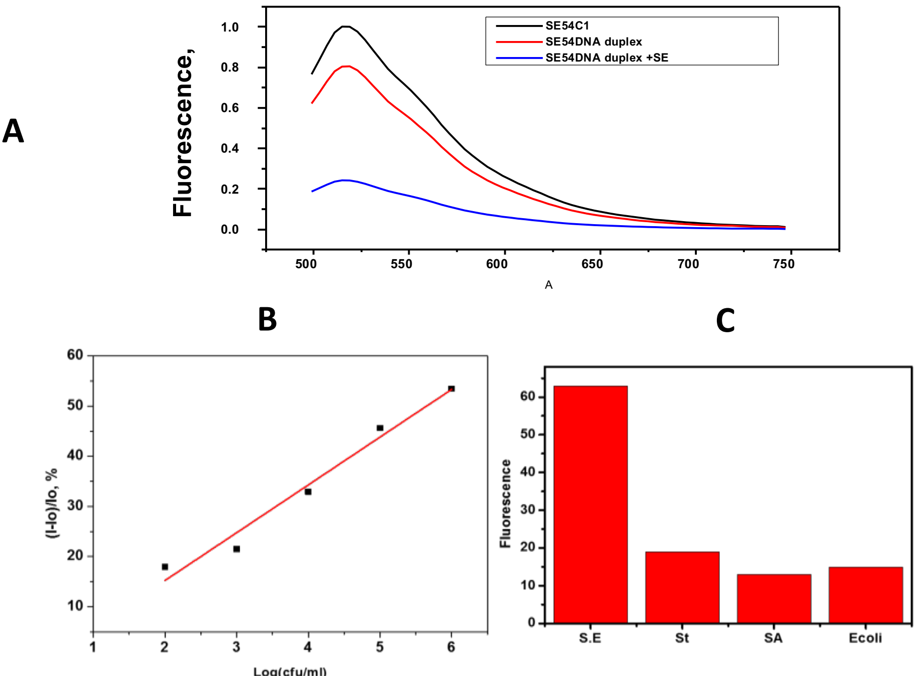


Fig.5.3: (A) The fluorescence spectra of SE54C1 (black), after formation of the duplex (blue), and after incubation with the bacteria (red). (B) Calibration curve, a plot of the change of the fluorescence intensity versus logarithm of the bacteria concentration. (C) The displacement sensor response against *S. enteritidis, Sa, E. coli and St*.

6.3.2. Determination of the dissociation constant of the SE54T aptamer

As shown from the displacement assay, the 29-mer truncated aptamer, SE54T (12-40), is involved in *S. entirica serovar enteritidis* binding and likely undergoes conformational change upon target binding. The percentage increase in the fluorescence intensity with increasing concentration of *S. entirica serovar enteritidis* cells was plotted in **Fig.** 5.4 to calculate the binding affinity of SE54T to *S. enteritidis*. The saturation curve obtained was used to calculate the K_d of the SE54T-SE complex by

non-linear regression. The K_d value was found to be 3.2 nM, which is two times higher affinity toward *S. entirica serovar enteritidis* compared to the original SE54 aptamer (K_d of 6.3), confirming that the truncated aptamer makes a more stable complex with *S. enteritidis.*

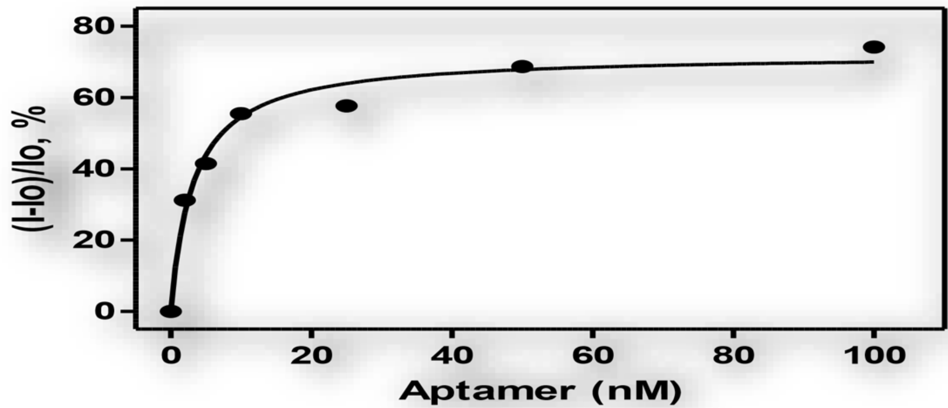
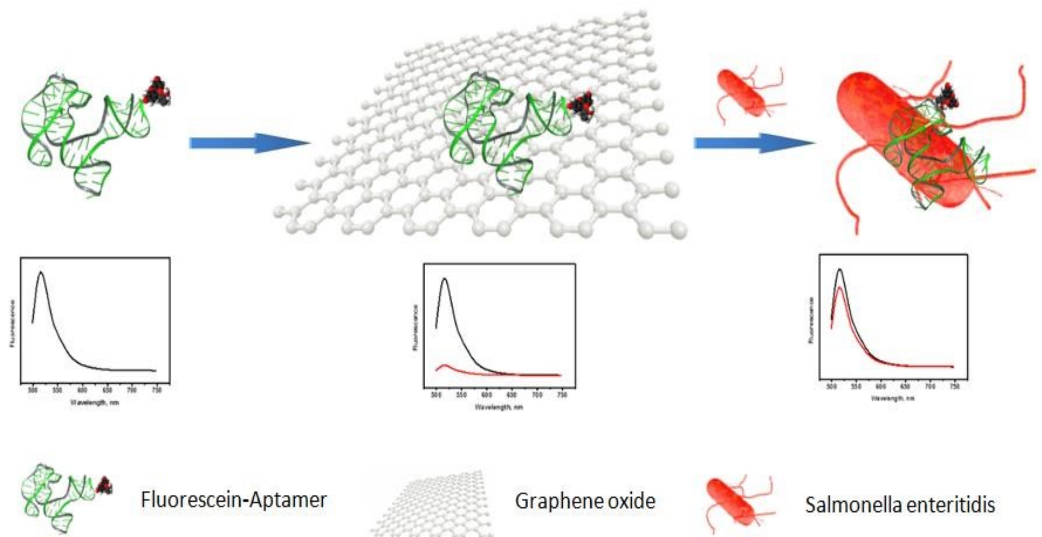


Fig. 5.4: Binding affinity curve of the aptamer SE54T, a plot of the aptamer concentration versus the percentage change in the fluorescent intensity.

6.3.3. Graphene oxide-based aptasensors

Since the truncated aptamer SE54T has shown a conformational change after binding to *S. entirica serovar enteritidis* as well as high affinity compared with the full aptamer sequence, it was chosen for the GO assay. As shown in **Scheme** 5.1, the GO assay is based on a simple fluorescence on/off strategy. The fluorescein labelled aptamers were adsorbed on the surface of GO by π-π stacking interactions. This adsorption leads to complete quenching of the fluorescence due to FRET from the fluorescein to GO in the absence of the target. However, in the presence of target molecule, the specific aptamer binds to its target and dissociates from the GO surface due to the conformational change of the aptamer, leading to a rapid increase in the fluorescence.



Scheme 5.1: **The working principle of the graphene oxide aptasensor.**

6.3.3.1. Optimization of the graphene oxide/aptamer ratio

With the aim to optimize the concentration of GO to give the maximum quenching efficiency of the fluorescence, different GO concentrations were initially investigated. The optimization experiments were performed for the full aptamer sequence SE54 and the selected truncated sequence SE54T. As shown in **Fig.** 5.5 A and B, in the absence of GO, a strong fluorescence peak of the fluorescein labelled aptamers was observed (red curves). The addition of increasing concentrations of GO solutions, ranging from 0 to 50 µg/ml, to the fluorescence aptamers lead to significant quenching of the fluorescence (more than 90% when the GO concentration reached 20 µg/ml). No further decrease in the fluorescence with the addition of a higher GO concentration was observed as shown in **Fig.** 5.5 C. This indicates an ideal off state (low background signal), therefore, the ratio of GO to SE54 or SE54T was

selected to be 20 µg/ml GO for 25 nM aptamer as the optimal conditions for further experiments.

6.3.3.2 Dose The response of the graphene oxide aptamer sensors changes with the length of aptamer?

The optimized GO/aptamer ratio was then used for the sensing experiment, comparing the behaviour of the full aptamer sequence with the truncated aptamer. As shown in **Fig.** 5.5 A and D, the off state of the fluoresceinaptamer/GO complex was turned on by the addition of *S. enteritidis*. The fluorescence intensity gradually increased with the increasing number of cells from 10^2 to 10^7 CFU/ml. To determine the LOD of the two aptasensors, calibration curves were plotted as the logarithm of the number of *S. entirica serovar enteritidis* cells vs % change in the fluorescence intensity as shown in **Fig.** 5.5 B and E. A linear relationship was observed for the two aptasensors with a LOD of 38 and 25 CFU/ml for the full length SE54 and SE54T, respectively. The LOD was calculated from 3 STD/m, where STD is the standard deviation of the aptasensors probe when no analyte was added, and m is the slope of the straight line. These results indicated that the truncated sequence gave better sensitivity compared with the full aptamer sequence. Moreover, the LOD of the truncated aptamer is almost two-fold less than the previously reported sensor fabricated with the full-length aptamer (40 CFU/ml). This is attributed to the improved affinity of the aptamer by removing the non-essential nucleotides from the parent aptamer, which can cause steric hindrance and decrease the affinity to the target.

6.3.3.3 Selectivity of the graphene oxide aptasensor

The selective binding of SE54TF and SE54F aptasensors to S. entirica serovar enteritidis cells was investigated by testing the sensor against other related bacteria, such as St, *Ec* and Sa. No considerable increase in the fluorescence intensity was observed by these pathogens, except St, compared to the response of S. entirica serovar enteritidis as can be seen in **Fig.** 5.6 C and F. The cross reactivity with S. entirica serovar enteritidis (25% increase in the fluorescence intensity) may be due to the similar structure of both species from the same family. Therefore, we believe that the developed GO-based fluorescence aptasensor represents a sensitive and selective platform for the simple detection of S. *enteritidis*. Moreover, this method has several advantages over other reported methods for the detection of S. entirica serovar enteritidis such as SPR (**Son** *et al.,* 2006; **Waswa** *et al.,* 2006), electrochemical immunosensors (**Melo** *et al.,* 2016; **Son** *et al.,* 2006), gold nanoparticle-based DNA sensor (**Alocilja** *et al.,* 2013) in terms of sensitivity, simplicity, capability of high throughput screening and multiplexing by introducing different fluorescent tags.

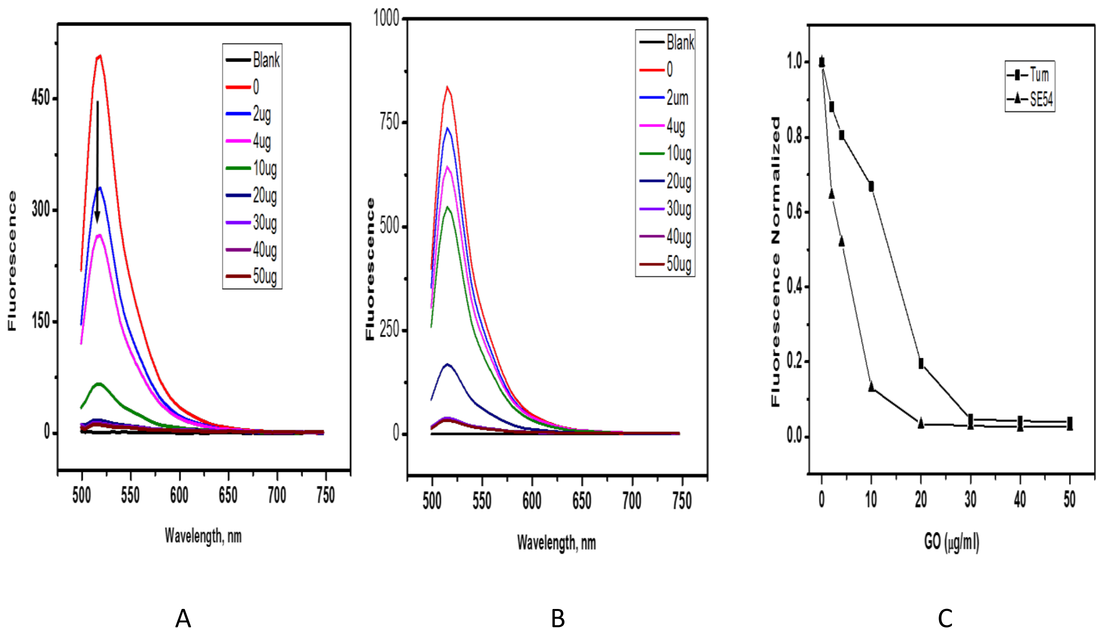
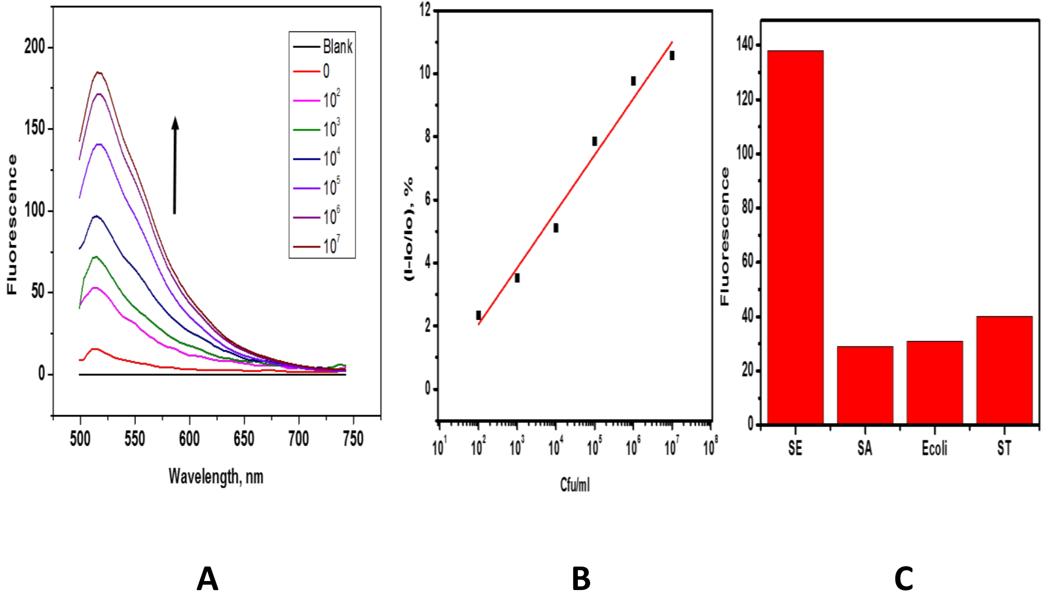


Fig. 5.5: The difference in behavior between the full-length and truncated aptamers. (A) The fluorescence spectra of 25 µM SE54 and (B) SE54T after incubation with different concentrations of GO. (C) Plot of the fluorescence intensity change versus the GO concentration.



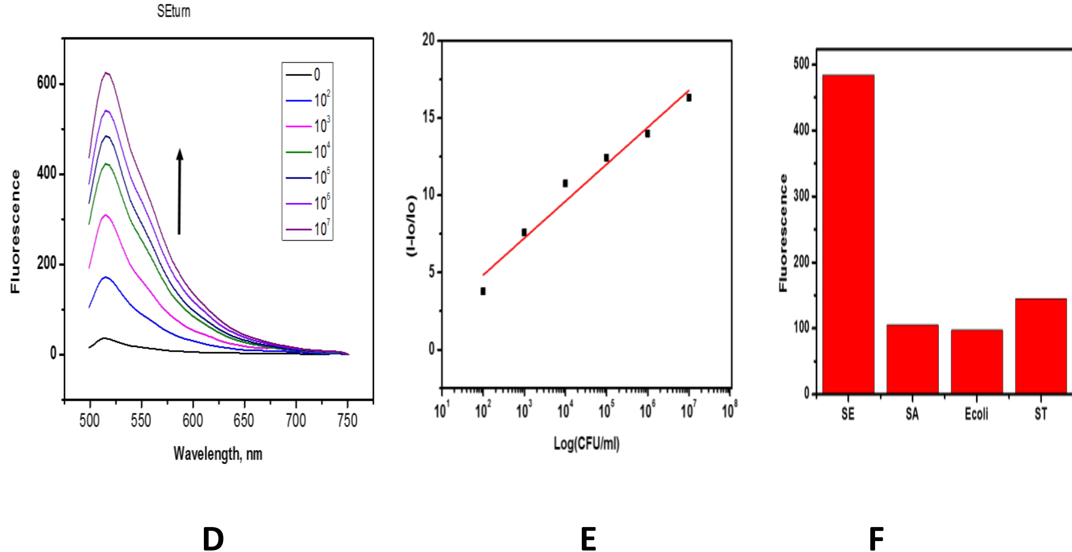


Fig. 5.6: sensitivity, linearity and specificity of both full-length aptamers. The fluorescence spectra of the SE54F/GO sensor (A) and SE54TF/GO sensor (D) after incubation with different concentration of bacteria. (B) and E are the calibration plots of the SE54F/GO and SE54TF/GO sensors, respectively. (C) and (F) are the selectivity studies for both GO aptasensors against Sa, Ec and St.

6.3.3.4. Testing of the SE aptasensor in spiked milk samples

The response of the SE54T aptasensor was tested for the detection of *S. entirica serovar enteritidis* from the real samples. In order to do that, 50 milk samples were spiked with 10^6 CFU/ml of *S. enteritidis.* The percentage recovery for each sample are tabulated in **Table** 5.1, showing the good recovery and indicating minimal interference from the milk matrix. This confirms the possible applicability of the developed assay for the detection of *S. entirica serovar enteritidis* in real samples.

Table 5.1: Application of the graphene oxide- based SE54T aptasensor in spiked milk Samples

Spiked CFU/ML	Found CFU/ML	% recovery	SD
1000	793	79.3	9.29
5000	4058	81.16	11.53
10000	7320	73.20	10.81

6.4. Conclusions

A mapping study was performed to identify a shorter aptamer sequence which binds with high affinity to *S. enteritidis*. Reducing the length of the aptamer by eliminating the non-binding region doubled the binding affinity and the truncated aptamer was then exploited in a GO-based competitive displacement fluorescence biosensor for the detection of *S. enteritidis*. The developed aptasensor based on the truncated aptamer showed a LOD of 25 CFU/ml, which was two times lower than the aptasensor fabricated using the full sequence. Moreover, the aptasensor showed high selectivity against other related bacteria such as *S. typhimurium*, *S. aureus* and *E. coli*. Our results suggest that the elimination of the non-binding region of the original aptamer by truncation leads to improvement of the binding affinity and consequently, less LOD for the sensor. Good recovery percentages of *S. entirica serovar enteritidis* were obtained from spiked milk samples using the developed aptasensor. In conclusion, this assay is simple to use, has

the capability of high throughput screening and can be multiplexed to detect other pathogens.

Chapter 7

Development of a colorimetric nanoparticle based assay for the qualitative detection of a loop mediated isothermal amplification (LAMP) amplicon of *Campylobacter jejuni* and *E. coli* 0157 in poultry processing plants

7.1 Abstract

This study aimed to develop an easy, fast and cost-effective method for the detection of pathogenic bacteria (*Salmonella spp.*, *E. coli.* 0157H and *Campylobacter jejuni*) in poultry processing plants using a combination of loop mediated isothermal amplification (LAMP) and nanotechnology. A lactoferrin-nanospheres composite was used as a biorecognition element, to collect negatively charged amplified DNA sequences on the surface of nanospheres (less than 50 nm) activated by carboxylic acid functional groups. Stainless-steel surfaces were artificially contaminated with different concentrations of bacterial cultures (10 to 10^8 CFU). The surfaces were then swabbed with the cotton swabs, which in turn, had been directly dipped in the DNA extraction solution. The samples in extraction solution were lysed at 95°C for 5 min in a heating block and then cooled on ice. The LAMP reaction was performed at 65°C for 40 min in the heating block. A dyed nanosphere solution (immobilized by lactoferrin) was added to each sample tube after amplification step and positive samples were visually detected by observing the aggregation of dyed nanospheres forming a disc near the top of solution, even after a little shaking; negative samples were characterized by dispersed dye in the solution. The results showed that the assay had very good sensitivity, which ranged between 10 CFU for both *Salmonella* and *E. coli.*, and 100 CFU in the case of *Campylobacter jejuni*.

7.2. Introduction

LAMP is an isothermal molecular technique used for gene amplification. Due to its high specificity, sensitivity and simplicity, it is considered as a valuable diagnostic tool in different fields like food safety analysis and medical diagnosis for the detection and identification of infectious diseases. This unique method depends on the use of four to six specially designed primers (two inner primers, two outer primers and two loop primers) targeting a number of sequences in the DNA strand. Amplification can be achieved in a single tube using a DNA polymerase enzyme dependent on the strand displacement activity, so there is no need for a denaturation step to obtain single stranded DNA (**Notomi** *et al.,* 2000). This is one of the techniques most important features which makes it more simple and applicable for many applications.

Furthermore, the detection and quantification of LAMP amplicons is one of the strengths of this technique. The process is simple and fast, does not need sophisticated equipment, and the products can be visually detected as a result of the turbidity from the precipitation of magnesium pyrophosphate ions during the amplification process. The turbidity can be also measured in real-time (**Abdullah** *et al.,* 2015) via the visual detection using various DNA dyes, such as like hydroxynaphthol blue (HNB), calcein and SYBR green. Quantification is possible by establishing a standard curve by plotting the known concentration of genes against the turbidimeter response.

In general, the LAMP test is usually achieved as a single tube technique (**Abdullah** *et al.,* 2015) by mixing all reagents required for the assay

(primers, dNTPs, buffer solution, template DNA) in a single tube and then isothermally incubation in a simple heating block or water bath at a temperature between
60°C and 65°C for about one hour when using only four primers. However, the process time can be decreased to be about 30 minutes when two loop primers are used, as they increase the efficiency of the amplification process. After incubation, the detection of amplicons can be achieved directly and there is no need for any further steps.

Despite the robustness of LAMP as a promising gene amplification technique, there are some limitations for its use in molecular applications. One of the most important limitations is the complex nature of the primer design, which is a critical step but has been overcome by the introduction of software for the design of LAMP primers. The availability of other materials required for the process is an additional limitation for using LAMP in molecular diagnosis.

7.3. Results and Discussion

7.3.1. Principle of the assay

Lactoferrin immobilized dyed nanobead composite was used for the detection of the LAMP product or amplified DNA. The lactoferrin molecules with a net positive charge act as a cross linker between the DNA sequences produced during amplification and the dyed nanobeads, causing aggregation of the dyed nanobeads in the form of coloured rings. Positive samples are differentiated from negative samples due to the

presence of coloured rings, with the dye particles dispersed throughout negative samples on gentle shaking as illustrated in **Fig.** 7.1.

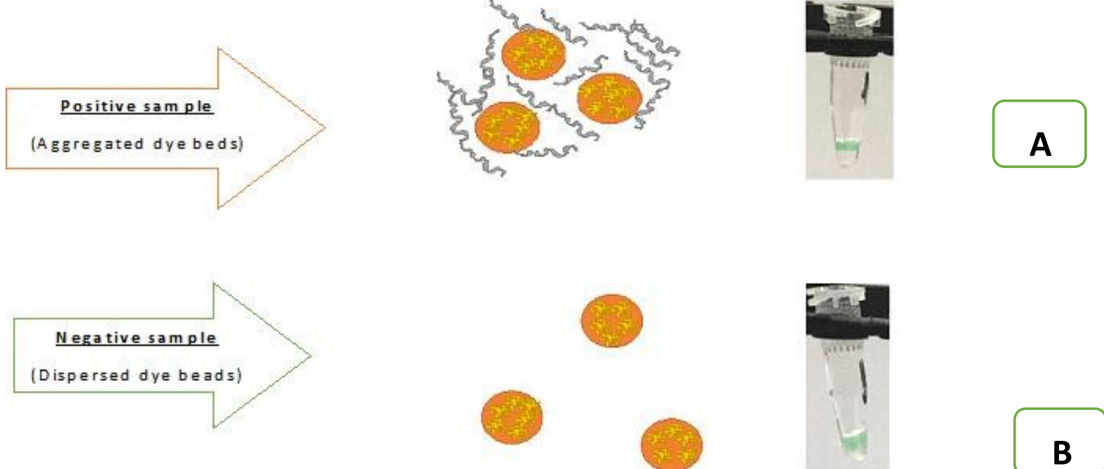


Fig. 7.1: Schematic diagram illustrating the principle of the assay. (A) Nanobeads surrounded by amplified DNA molecules results in the formation of dense colour rings near the surface of positive samples. (B) Orange nanobeads without DNA molecules are distributed throughout negative samples.

7.3.2. Simplicity of the assay

Both the extraction and amplification processes in this experiment were achieved within 45 to 60 minutes, due to the high efficiency of loop primers which possess the ability to accelerate the amplification process. Furthermore, the assay only required a very simple and cost effective tool, a simple heating block was enough to achieve successful LAMP. The detection of amplified DNA sequences using our lactoferrin immobilized dyed nanobead composites did not require any instruments for the detection of positive samples in comparison to the original technique, which required a turbidimeter to visualize the aggregation of the dyed particles.

7.3.3. Sensitivity of the assay

A serial dilution of the bacterial cultures tested (ranging from 10^8 to 10 CFU/ml) was used to evaluate the sensitivity of the assay by determining the LOD. Also, a negative control was used to confirm reliability of the results.

7.3.4. Detection of the *Campylobacter* amplicon

Green dye coated polymer nanobeads immobilized with lactoferrin were used to detect amplified *Campylobacter* gene sequences in the concentration range from 10^8 to 10 CFU/ml. After amplification, 5 µl of the dye bead solution was added and the tube was gentle shaken to disperse the dye particles in the solution in the case of a negative sample, whereas in positive samples containing DNA sequences, ring shaped green aggregates were formed. This procedure gave very good results up to a concentration of 100 CFU/ml as shown in **Fig.** 7.2. The results were comparable with a previous study by **Yamazaki** *et al.* (2008), which reported a LOD for *Campylobacter* of 20-200 CFU/ml using a LAMP technique.

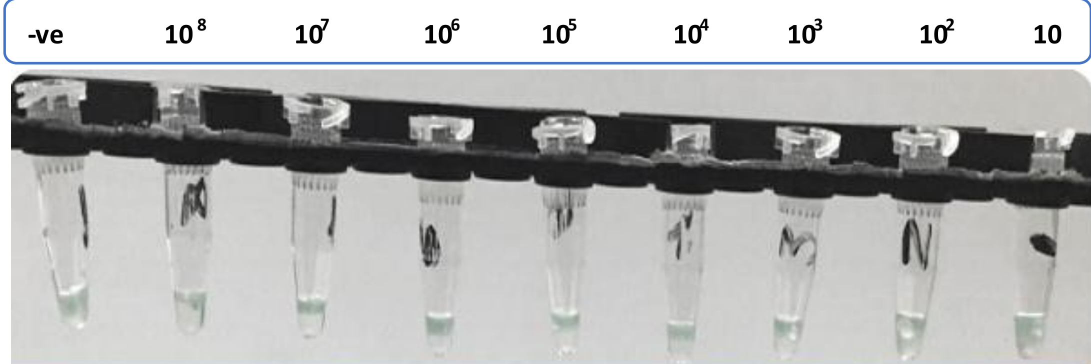


Fig. 7.2: Serial dilution of *Campylobacter* jejuni showing the formation of the nanobead aggregates during LAMP.

7.3.5. Detection of the *E. coli* 0157 amplicon

Yellow coloured dyed nanobeads were used to detect *E. coli* 0.157 DNA sequences as described previously for *Campylobacter.* The LOD for *E. coli.* was determined in the same way and found to be 10 CFU/ml as shown in **Fig.** 7.3. The obtained results are comparable with the results from the previous studies, as discussed in **Table** 7.1.

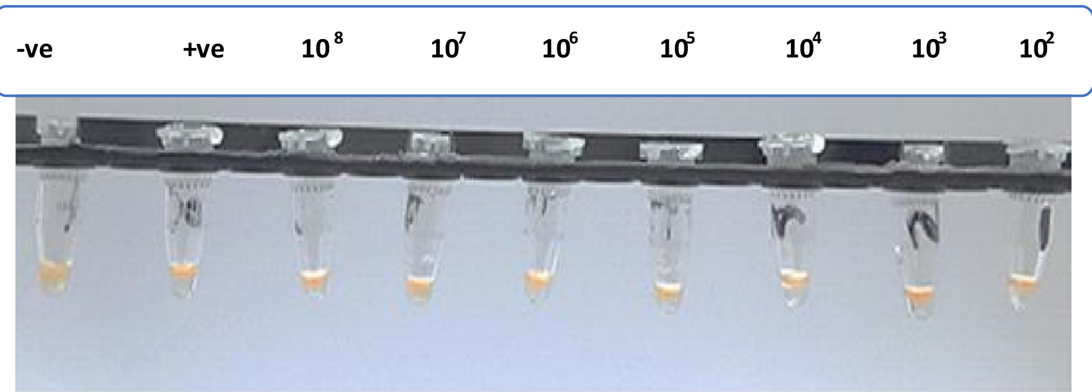


Fig. 7.3: Serial dilution of *E. coli.* 0157 showing the formation of the nanoparticles aggregates during LAMP, positive samples can be identified by the bright yellow disks of nanoparticles on the top of solution.

Table 7.1. Overview of LAMP assays for bacterial detection

Pathogen		Method	Matrix	LOD	Reference
Salmonella	spp.	LAMP[a]	Cilantro (coriander leaves), lettuce, parsley, spinach, tomato, jalapeno.	2 CFU 25 g	Zhang *et al.,* 2011
	spp.	LAMP	Milk	10^2 CFU/ml	Zhu *et al.,* 2008
	spp.	LAMP	Raw milk	10^8 CFU/ml	Wang *et al.,* 2008a
	spp.	PMA[e] LAMP *in situ* LAMP	Cantaloupe, spinach, tomato Eggshell	6.1 103/26.1 10^4 CFU/g 1 CFU/cm	Chen *et al.,* 2011a Ye *et al.,* 2009, 2011

	spp.	LAMP kit	Poultry, livestock, other raw meat, dairy products	10^2 CFU/ml	He *et al.,* 2010
	enterica	LAMP	Liquid egg	2.2 CFU/test tube	Hara-Kudo *et al.,* 2005
	Enteritidis				
	Typhimurium	Reverse transcription LAMP	Pork products	10^2 CFU/25 g	Techathuvanan *et al.,* 2010
	Typhimurium	Real-time reverse transcription LAMP	Pork processing environment	10 CFU/ml	Techathuvanan *et al.,* 2011
	Typhimurium	LAMP	Fresh pork, whole chickens, green vegetables	16 CFU/reaction	Zhang *et al.,* 2012
	O9 serogroup	LAMP	Chicken	10^3 CFU/ml	Okamura *et al.,* 2008
	O9 serogroup	LAMP	Meat, milk	35 CFU/250 ml	Li *et al.,* 2009
	serotype D	LAMP	Chicken meat	10 CFU/reaction	Ravan & Yazdanparast, 2012
Escherichia coli	Spp.	LAMP	Raw milk	410 CFU/ml	Wang *et al.,* 2009b
		LAMP	Raw milk	440 CFU/ml	Wang and Huo, 2012
		LAMP	Pork meat	10 CFU/ml	Jiang *et al.*, 2012
		LAMP	Meat	1.8 CFU/g	Liu *et al.,* 2011a

7.3.6. Advantages of the developed assay

The comparison of the current detection method developed in this work with the original method based on the colour change of reaction solution, indicates the simplicity and accuracy of the new assay. The simplicity and accuracy of the assay are due to the formation of an easily recognizable disc of coloured nanoparticles near the surface of the reaction solution. This will avoid confusion associated with the colour change, which can be difficult in some cases as shown in **Fig.** 7.4.

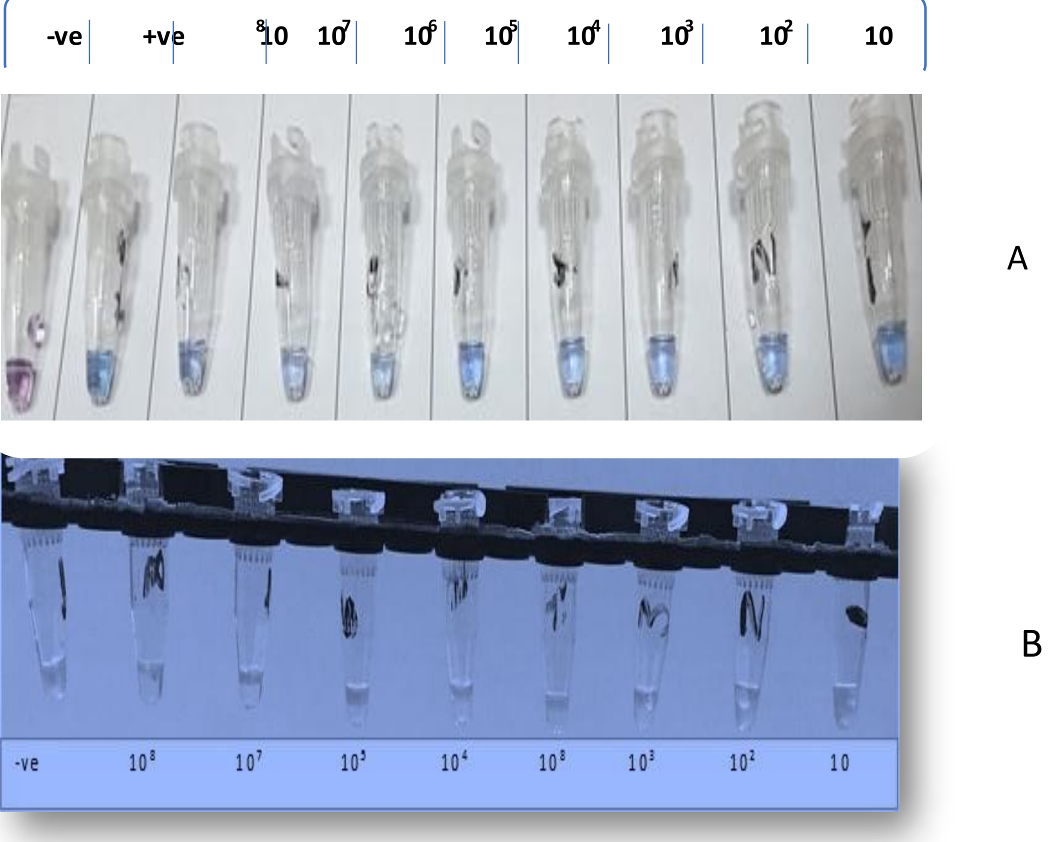


Fig. 7.4: Comparison of the original detection method in LAMP depending on the colour change from purple to faint blue (A) and the newly developed method which depends on the aggregation of nanoparticles in the form of a green disc near the surface of the reaction solution (B).

7.4 Conclusion

The DNA detection method developed in this work is rapid, simple, reliable, and cost effective using lactoferrin immobilized dyed nanobead composites as a substrate to detect amplified DNA sequences produced during LAMP. The identification of positive samples is a simple process and does not require any sophisticated instruments, as it can be achieved visually by observing the coloured ring in the reaction solution formed by the aggregation of dye particles. The method was used to detect *C. jejuni* and *E. coli* 0157*,* as representative food poisoning bacteria on stainless-steel surfaces widely used in poultry processing plants. The LOD was in the dynamic range of 10 and 100 CFU/ml for *E. coli.* 0157 and *C. jejuni* respectively, which were comparable with reported values. In conclusion, this technique is suitable for bacterial detection in food safety programs.

Chapter 8

Conclusions and future work

8.1. Conclusion

The work presented in this study involved a number of improvements to three of the main techniques used for detecting bacteria and viruses in food in order to increase efficiency and decrease cost and time. In chapter three, the detection step of the immunoassay method was modified by the use of dyed nanobeads, reducing the incubation time to only one minute for the target bacteria on the surface of cotton, then allowing the immediate colorimetric detection. The developed assay was used for screening four bacteria in three surfaces. In the newly developed assay, a specific antibody immobilized on cotton swab was used to pre-concentrate bacteria for the surfaces, and then it was complexed with another specific antibody conjugated with dyed nanobeads. Sensitivity of the assay was tested on the concentration range of (10 to 10^8 CFU/ml), with a very low detection limit which reached 10 CFU/ml. the results of the assay were confirmed by using real-time PCR analysis. The linearity of the color intensity and concentration of cells in samples means the, it can be used not only for qualitative analysis but also for semi-quantitative analysis. The main advantage of this assay it the simplicity which enable non-skilled personnel to do it in addition to being instrument-free. So it can be suitable for onsite analysis.

The immunoassay technique was further improved by the use of a general recognition element, lactoferrin, for all bacterial species used in this study as demonstrated in chapter four. In this assay, the activated cotton swab were coupled with lactoferrin which was used for pre-concentration of bacterial cells form different contaminated surfaces

including stainless steel, glass, and chicken meat surface. Then, the pre-concentrated cotton swab was immersed in the developing solution consisting of different coloured polymer nanobeads which were immobilized with specific antibody for each pathogen. The detection limit of the assay ranged between 10 to 1000 CFU/ml and all results were confirmed by LAMP as a confidential molecular technique. The modified immunoassay is very simple and cost effective in comparison to the traditional immunoassay techniques. The same assay was used to screen norovirus in three types of samples including lettuce, cucumber and chicken meat and it gave a very good sensitivity as explained in chapter five.

A graphene oxide-based biosensor was developed in chapter five using a truncated aptamer as a recognition element and graphene oxide film as a quenching surface. In this a fluorescence-based study, the highest affinity truncated aptamer from the full-length was mapped. Molecular beacon and displacement assay design were used to identify the best truncated aptamer. The selected truncated aptamer was used to develop a simple fluorescein-based graphene oxide sensing platform in which, the fluorescein labelled aptamer was adsorbed on the G.O surface leading to quenching the fluorescein intensity. However, in the presence of target bacteria the labelled aptamer were released from the G.O surface forming a stable complex with target bacteria. This technique allowed higher sensitivity than using traditional quenching groups and the aptasensor fabricated using truncated aptamer gave higher sensitivity than the full-length aptamer with detection limit two times lower than the full-length.

An improvement in the detection of the LAMP product was achieved in chapter seven. The modified approach depends on the ability of lactoferrin molecules to collect negatively charged DNA molecules. In this assay, the DNA was extracted and amplified from artificially contaminated swabs using commercial LAMP kits. Then dyed nanobeads immobilized with lactoferrin was added to the amplified samples. This composite of lactoferrin and dyed nanobeads causes the aggregation of DNA molecules. This aggregation can detected visually by observing the formation of colored disc on the top of amplification tube. This technique has advantage over the original LAMP technique which depends on the color change. The assay was used to detect campylobacter and E.coli 0175H7 and it gave detection limit of 100 and 10 CFU/ml for the two bacteria respectively.

8.2. Future work

In the future work, the scope of the developed immuno-based screening kits will be extended to include more bacterial species, viruses, toxins and other biomolecules. Also, additional validation studies will be applied for the use of different materials as substrates for fixing nanobeads other than cotton buds which are currently used to for the biorecognition element, the main component of the detection process. Proposed materials include paper and some types of polymer which may be more suitable for other applications such as medical diagnosis as well as for environmental and food inspection, which require ready kits for immediate and on-site screening of microbial contaminates. The next

step in this research will focus on fabricating detection strips for these applications, as well as the design of multitarget screening kits or how to detect more than one species using the same screening kit. Preliminary experiments revealed some difficulties in identifying colour, so the multitarget screening assays require further improvement and optimization. Furthermore, the stability of the beads on the cotton buds for the multitarget kits will be studied under different storage conditions to determine the effective life-span of the kit.

For the LAMP assay procedure, a new detection technique will be developed using lactoferrin, in which the LAMP product will be mixed with a lactoferrin solution to aggregate the DNA molecules. These aggregates could then be detected on the surface of filter paper in the form of black spots. Once the procedure has been optimized for nanobead concentrations, the developed assay will be applied for other applications, such as the detection of human parasites, fungi and viruses.

For the graphene oxide-based aptasensor, a selected truncated aptamer was used to develop a simple fluorescence-based graphene oxide sensing platform. In this sensing platform, the fluorescein labelled aptamer was adsorbed on the GO surface by π-π stacking interaction leading to quenching of the fluorescence. In the future work, a similar aptasensor will be developed for detecting other biological toxins, like aflatoxin and bacterial toxins. Due to the high specificity of aptamers, they can be used to develop sensing platforms for detecting chemical contaminants like antibiotics in meat and pesticide residues in food and biological samples. The analysis of biomarkers in blood, urine and other body fluids is one of

the methods applied in the early detection of diseases and the GO-based aptasensors developed in this work have the potential to be useful for the detection of biomarkers, such as thrombin and immunoglobulin (Ig). They could also be used for the identification of cancer markers, such as lymphoma (Ramos) cells and leukaemia. Identification of these cancer markers requires specific probes to bind in order to identify potential risk factors, such plasma proteins or free DNA in blood cells (**Hong** *et al.,* 2012). Indeed, recent studies have successfully used aptamers for targeting tumor markers.

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