

## ABSTRACT

Title of Document: Identification of monoclonal antibodies to be used in Biosensors to detect *Listeria monocytogenes* and *Salmonella*

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Food industry needs quick and reliable methods to make sure the food supply is not contaminated with pathogens. Monoclonal antibodies specific to *Listeria monocytogenes* and *Salmonella* were screened to choose the ones most suited for biosensor development to detect these bacteria in foods. Out of the eight antibodies procured for *L. monocytogenes*, C11E9 was able to recognize both *L. monocytogenes* and *L. innocua* with no cross-reactivity to any of the gram-negative bacteria tested. It however reacted with a *Bacillus* strain and a *Staphylococcus* strain. For the monoclonal antibodies specific to *Salmonella* sp., IFR0111 reacted with 48% of the *Salmonella* strains tested, that included 20 different serotypes. It did not cross-react with any of the gram-negative strains tested but showed reactivity to a *Staphylococcus aureus* strain. Further studies are required to test the sensitivity of these monoclonal antibodies to correctly determine their potential to be used in biosensors.

IDENTIFICATION OF MONOCLONAL ANTIBODIES TO BE USED IN  
BIOSENSORS TO DETECT *LISTERIA MONOCYTOGENES* AND  
*SALMONELLA*

By

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## **I OBJECTIVE**

The objective of this study was to test monoclonal antibodies specific to *Listeria monocytogenes* and *Salmonella spp.* The potential of using these antibodies in biosensors for detection of these bacteria in the food would be explored.

## **II INTRODUCTION**

Consumption of food contaminated with microbes, toxic chemicals or any other hazardous material can lead to food-borne illness. According to the Centers for Disease Control and Prevention, food-borne diseases cause approximately 76 million illnesses, 325,000 hospitalizations, and 5,200 deaths in the United States each year. Nearly 14 million illnesses, 60,000 hospitalizations, and 1,800 deaths are caused by known pathogens<sup>1</sup>. It is difficult to estimate the global incidence of food borne diseases, but it has been reported that in 2000 alone 2.1 million people died from diarrhoeal diseases because of the consumption of contaminated food and water<sup>2</sup>. Many of these food borne illnesses are not even reported, but they have economic impact because of medical costs, missed work hours etc. Massive out breaks take their toll not only on the health of people but also on the economy.

Foods of animal origin are reported to be the cause of most of food borne illnesses. Farm animals if infected by any of the pathogens can pass the infection to other animals or the people handling them. However, processing intended to increase the shelf life of food may lead to cross contamination. As mentioned by Stephen Knabel, a food scientist at Pennsylvania State University, “only 5% of

live poultry are contaminated with *Salmonella*, but after processing, nearly half of the carcasses contain *Salmonella*.” Processing steps like defeathering, slaughtering, chilling, and processing provide opportunities for cross-contamination. There could be extensive contamination if there is accidental puncturing of the intestinal tract during slaughter<sup>3</sup>.

Changing lifestyles have made people more dependent on ready to eat foods that are longer lasting and good to taste. So people today depend on others for the safety of food they consume. The food processors can have monitoring mechanisms to make sure that the food they are distributing is not contaminated because once the contaminated food is distributed, the only option left for the processor to prevent the spread of food borne illness is to go for product recall. Recall makes the food processor to incur economic losses and customers may also lose confidence in their product and refrain from buying the same product again. Recalls can be as serious as causing the company to shut down<sup>4</sup>.

Therefore, it is very important for the food manufacturers to have a powerful and reliable monitoring system for the incoming ingredients, food processing equipment and environment and the finished product to make sure that there is no contamination at any step. The traditional microbiological methods used to test the food for specific pathogens are time consuming and laborious. Rapid and easy methods for detection of pathogens can save the food processors by timely identification of the problem and save them from serious consequences of a product recall.

Various kinds of molecular, immunological and spectroscopic rapid methods have been tried in food analysis. Optimum method would be the one that is rapid and has sensitivity and specificity along with being economical to be used in industry.

## **2.1 *Listeria monocytogenes***

### **2.1.1 Classification**

*Listeria monocytogenes* is a member of the Family *Corynebacteriaceae*, order *Eubacteriales*. The genus *Listeria* consists of 7 species but *L. monocytogenes* has been implicated as the causative agent in several foodborne outbreaks and is responsible for Listeriosis. The species name *monocytogenes* is derived from the fact that a number of monocytes are found in the blood of infected mono-gastric animals. It is a gram positive and nonsporeforming, bacteria. It is rod shaped with rounded ends measuring 1.0 to 2.0 by 0.5 $\mu$ . It is catalase positive, oxidase negative and expresses a  $\beta$ -hemolysin<sup>5,6</sup>. Catalase and Oxidase are the enzymes whose presence or absence in the bacteria is analyzed for bacterial identification. The cell wall consists of a thick homogenous structure that surrounds the cytoplasmic membrane. Dry cell wall consists of about 35% peptidoglycan, the remaining cell wall consists of teichoic acids which are polymers covalently linked to a specific site on the peptidoglycan. Structurally there are two types of teichoic acids that exist amongst *Listeria* serotypes. One type with ribitol residues covalently linked by phosphodiester bonds and sometimes N-acetylglucosamine at C-2 are found in serotypes 1/2a, b and c, 3a, b, c and 7. The other type has N-acetylglucosamine integrated into the chain and

is found in serotypes 4a, b and d. Majority of the cases of human Listeriosis are caused by 1/2a, 1/2b and 4b serotypes<sup>10</sup>. The organism has peritrichous flagella but it occurs within a narrow range of temperature. When the organism is grown between 20 and 25 °C, the flagellin is produced and assembles at the cell surface but flagellin production is reduced at 37 °C<sup>5</sup>.

### **2.1.2 Growth conditions**

*Listeria monocytogenes* is a facultative anaerobic rod that grows between –0.4 to 50 °C. Minimum pH required for the initiation of growth ranges from 5.0 to 5.7 at 4 °C and from 4.3 to 5.2 at 30 °C<sup>5</sup>. But it can grow from a pH range of 5.0 to 9.6. It can also multiply in high salt (up to 10% sodium chloride) or bile concentrations. It requires biotin, riboflavin, thiamine, thioctic acid and some amino acids for growth<sup>9</sup>. When grown anaerobically, *Listeria* spp. use hexoses and pentoses, but under aerobic conditions, maltose and lactose are also utilized for growth. *L. monocytogenes* use glucose, lactose and rhamnose for growth under aerobic conditions<sup>5</sup>.

### **2.1.3 Distribution**

*Listeria monocytogenes* can be a normal resident in human intestine. 5 to 10% of the population can be carrier of *L. monocytogenes* at any time. It is widely present in plant, soil and surface water samples. *L. monocytogenes* also has been isolated from cattle, sheep, goats and poultry<sup>5</sup>. Infected animals can shed them in faeces, milk and uterine discharges. *L. monocytogenes* causes abortion in pregnant women and as a result this bacteria can be found in aborted fetus. Only recently this bacteria has emerged as a major food borne pathogen.

Because of its ability to survive at low temperatures, there is high incidence of *L. monocytogenes* in Ready to eat (RTE) foods. Presence of this bacterium in food processing plants may result in human outbreaks and food recalls. USDA has issued a zero tolerance policy for *L. monocytogenes* because of increased food borne illnesses from consumption of *L. monocytogenes* contaminated meat and poultry products. Various food products like sliced ham, luncheon meats, salads, spreads, uncured cooked poultry, jerky etc were found to be contaminated by *L. monocytogenes*. Potential sources of contamination in food processing plants can be raw products and ingredients, chilling solutions, loose products, rework etc. There could be post-processing contamination from surface areas of the plant, equipment, and packaging material<sup>8</sup>.

#### **2.1.3.1 *Listeria monocytogenes* in the environment**

*Listeria monocytogenes* has been reported to be widespread in the natural environment. It has been isolated from soil, vegetation and water. Weis and Seeliger, (1975) suggested this bacteria to be a saprophyte that lives in a plant and soil environment, thus contracting human and animal via various routes. They sampled a wide range of environments including cultivated fields, uncultivated fields, forests, wildlife feeding grounds, mud from creeks and rivers and also faeces and residues of fodder from the wildlife feeding grounds. The highest incidence of these bacteria was found on surfaces of soil and plants, especially in the fields that had been fallow for years and were overgrown with grass and shrubs. They also reported *L. monocytogenes* in faeces of animals, old moldy samples of fodder residues and mud. The lowest number was reported

in cultivated fields. Distribution in forests was found to be rather scattered. 1/2b and 4b were the predominant serovars for the isolated strains<sup>11</sup>. A study conducted on the fields that had been planted the previous year. Seven out of twelve fields were found to have *L. monocytogenes*. Most of the strains recovered were non-pathogenic<sup>12</sup>. Surveys on farm vegetation suggested that 10-30% crops had *L. monocytogenes*. It is reported to grow quickly on vegetation stored at low temperature (2 –5 °C). Survival on vegetation is also reported to be plant dependent. Carrots have lower incidence of *L. monocytogenes* as compared to other roots crops because of anti-microbial compounds<sup>13</sup>.

It was reported that incidence of *L. monocytogenes* was more in soil than in animal feed, and thus soil can be the source of animal feed contamination. High prevalence of *L. monocytogenes* was found on ruminant farms, thus suggesting that they can be an important natural reservoir for this bacteria. The subtypes of bacteria obtained from the farms overlapped with those responsible for human Listeriosis<sup>21</sup>.

Studies have reported that application of sewage sludge for the fertilization of farmlands increases the risk of *L. monocytogenes* contamination. Sixty% of the sewage samples tested were found to be positive for *L. monocytogenes*. Most of the isolates belonged to serotypes 4b and 1/2, i. e. the ones responsible for human Listeriosis<sup>14, 15, 16, 17</sup>.

Badly prepared silage, i.e. the one that has not been fermented properly, provides favorable environment for the survival and growth of *L. monocytogenes*. This is a source of infection for the animals that consume the silage. Higher pH

in poorly prepared silage can lead to survival and proliferation of *L. monocytogenes*. A pH lower than 5.0 has been reported to kill or inhibit these bacteria<sup>13, 18</sup>.

*Listeria monocytogenes* is also reported to be present in water bodies like ponds and river water. Sixty-five isolates were obtained from a group of aquatic animals tested in 1968 and five isolates were obtained from a group of animals tested in the following year. It was hypothesized that the incidence of this bacteria was more in the ponds used by deers<sup>19</sup>. A survival study conducted on *L. monocytogenes* in water and soil suggested that it survived for eight weeks in pond water without multiplication. Growth rate in soil was correlated with ambient air temperature, with major multiplication occurring in late winter and early spring<sup>20</sup>.

### **2.1.3.2 *Listeria monocytogenes* in animals**

It is found in mammals including domestic and feral ruminants and also monogastric animals. It affects sheep, goat, cattle and water buffalo. The disease in sheep and goat is more acute causing death to occur within 4 to 48 hrs, but that in cattle is chronic, and more cows survived. It is also found in monogastric animals including swine, some house pets like dogs, cats and squirrel. Domestic rodents like rabbit and chinchillas are also reported to be infected by *L. monocytogenes*. It is also found to infect feral animals like rabbits and deer with no particular preference to domestic or feral animals<sup>6</sup>. These animals can also act as carriers of this bacteria. The relation between human Listeriosis and exposure to these animals or through indirect contact cannot be ruled out. Case

reports of human Listeriosis caused by consumption of un-pasteurized milk from cows infected with the bacteria have been reported. This also helped in establishing *L. monocytogenes* as a food-borne pathogen<sup>10</sup>. Another study reported that rats had the highest carrying rate for *L. monocytogenes* as compared to dogs, cattle and pig. They also carried the highest percentage of 1/2a, 1/2b and 4b serotypes responsible for human Listeriosis as compared to dogs, cattle and pigs<sup>102</sup>.

Epidemiological characteristics of *L. monocytogenes* were found to be different between bovine and small ruminant farms. There was higher prevalence of *L. monocytogenes* without Listeriosis in bovine farms as compared to small ruminant farms. This could be because the diversity of *L. monocytogenes* populations on bovine farms was much higher than in small ruminant farms that had one or a few subtypes. Higher prevalence of these bacteria was found in faeces than the feed thus indicating that animals exposed to *L. monocytogenes* contaminated silage were able to amplify the bacteria. On the other hand, on small animal farms, incidence of *L. monocytogenes* was more in feed than in faeces, thus indicating that the small ruminants are less likely to amplify the ingested *L. monocytogenes*<sup>21</sup>.

### **2.1.3.3 *Listeria monocytogenes* in birds**

At least seventeen avian species were reported to harbor *L. monocytogenes*. It has also been isolated from the spleen of apparently normal chickens. Chicken, Geese and turkey are reported to be infected by this bacteria<sup>6</sup>. A study conducted on indigenous domesticated chickens in Kenya

reported that chickens are the carriers of *L. monocytogenes* and other *Listeria* species<sup>22</sup>. 17.3% of the birds tested in a study were found to be *Listeria* positive. It was also suggested that the birds played a role in spreading *L. monocytogenes* after their isolation from the shrub leaves<sup>11</sup>.

#### **2.1.3.4 *Listeria monocytogenes* in fish**

*Listeria monocytogenes* was found in fish and crustaceans. It was not proved that crustaceans carried *L. monocytogenes* because it was also isolated from the water in the stream, but it was suggested that aquatic life can carry and spread it<sup>6</sup>. Aquatic animals like snails, leech, turtle and frogs were reported to carry *L. monocytogenes*. Though no relation could be established, it was hypothesized that *L. monocytogenes* was more prevalent in ponds frequently used by deers, and deers were reported to carry it in faeces<sup>19</sup>.

#### **2.1.4 *Listeria monocytogenes* as foodborne pathogen**

Though there was information available about the direct transmission of *L. monocytogenes* infection from infected animals to farm workers and others coming in contact with these animals, it was only in the early 80s that food was identified as a carrier of this bacteria, thus causing epidemic and sporadic cases of Listeriosis. In 1979, twenty cases of Listeriosis caused by serotype 4b occurred, but they were not reported until several years later. The foods responsible for this outbreak were suggested to be raw vegetables served with the meals. In the year 1981, forty-one Listeriosis cases were reported in the Maritime Provinces of Canada with 28.6% adult mortality rate. The cause of outbreak was found to be coleslaw that was grown in a farm fertilized with sheep

manure. There were reported cases of sheep dying because of Listeriosis in 1979 and 1981. Also this cabbage was stored in a cold storage at the farm, which might have helped this psychotropic bacteria to survive and grow<sup>5,9</sup>.

Forty-nine cases of Listeriosis were reported in Massachusetts in 1983. Pasteurized whole milk or 2% milk was suggested to be responsible for the outbreak. The raw milk used by the facility that processed this milk was reported to have *L. monocytogenes*. This raw milk was procured from bovine farms where four cases of bovine Listeriosis had been reported in that year. Investigation of the plant did not show any evidence of improper milk pasteurization. Also skim milk prepared from the same raw milk and processed in the same facility on the same day was not reported to have *L. monocytogenes*. Questions were raised about the adequacy of epidemiological investigations and it was doubted that milk could be the reason of the outbreak. On the other hand another hypothesis was a possible increase in heat resistance of *L. monocytogenes*<sup>5,9</sup>.

In 1985, in California Mexican style soft cheese was found to be responsible for an outbreak that affected eighty-six people. The mortality rate was 34%. Phosphatase test is used to determine if pasteurization has been done properly. The sample is tested for the presence of alkaline Phosphatase, an enzyme that is inactivated by pasteurization temperature. Cheese was subjected to Phosphatase test to ensure proper pasteurization, and the results suggested that cheese was Phosphatase positive and that there was improper pasteurization. However, the adequacy of Phosphatase test on Cheese has been questioned. No *L. monocytogenes* was reported in the raw milk samples taken

from farms that supplied the Cheese manufacturing facility. So it was suggested that there was a possibility of post-pasteurization contamination. Another outbreak in Switzerland was also suggestive of a soft surface ripened cheese to be culprit<sup>5, 9</sup>.

There has been many recalls because of reported cases of Listeriosis or as precautionary measures taken even when there were no reported cases. Several brands of Brie cheese were recalled in 1986 in France based on continued testing of products by the regulatory agencies. Kraft Inc. voluntarily recalled 25,000 cases of ice-cream bars as a precaution after detecting the organism during routine testing. Liederkranz cheese was recalled in 1985, and in Arizona, soft Mexican style cheese was recalled in 1986<sup>5, 9</sup>. These reports where *L. monocytogenes* from food was found to be responsible for human Listeriosis established *L. monocytogenes* as an important food-borne pathogen.

Many countries have established the limits tolerable levels of these bacteria in Ready-to- Eat foods because of its ability to survive and grow in refrigerated conditions. Countries like US and Italy have imposed zero tolerance that means absence of any *L. monocytogenes* in 25 grams of the sample. Some countries have risk-based approach, so they have a zero tolerance for some foods and below 100 colony forming units (cfu)/gram for others. While in countries like France, Germany and Netherlands, the tolerance level is below 100 cfu/g at the time of consumption<sup>23</sup>.

Recontamination is another route of this pathogen to enter the food. Recontamination of an otherwise clean product can occur from contaminated raw

material coming in contact with finished product, or contact with contaminated food contact surfaces, food processing environment and last but not the least, during handling and distribution<sup>25</sup>. So, in the food processing plants, *L. monocytogenes* pose a very serious problem. Though meat and dairy products are more notorious to harbor the bacteria, vegetables and fruits have also been reported to carry them. The processing plant can be contaminated by the raw material coming in the facility, e.g. if the fruits or vegetable surfaces are contaminated with manure containing the bacteria, they could lead to contamination of the processing plant and in turn the product made from the fruits or vegetables. *L. monocytogenes* can also adapt to some microenvironments in the processing plants like some crevices or drains etc, which are hard to clean. And this may lead to occasional contamination of raw or finished product<sup>18</sup>. *L. monocytogenes* is also reported to form biofilms and survive for prolonged periods of time on the surfaces of food processing equipment<sup>24</sup>.

#### **2.1.4.1 *Listeria monocytogenes* in Dairy products**

Milk and milk products have been reported to be susceptible to *L. monocytogenes* contamination. Contaminated raw milk is an important source of contamination of the processing plants and the finished products. There are many factors that can contribute to raw milk contamination. Exposure of milk to faeces contaminated with *L. monocytogenes* can lead to raw milk contamination. Poorly prepared silage has been reported to be a source of faecal contamination. Poor cow housing and milking hygiene are other factors that can contribute to

raw milk contamination. The incidence of *L. monocytogenes* also shows a seasonal pattern. It is highest in the winter months and low in hot weather months. This seasonal variation can be due to a variety of reasons including feeding practices, herd management or a change in some unknown factors that might affect the bacteria-animal relationship or bacteria-environment relationship<sup>26, 27</sup>. In a study conducted in France over a period of four years, the average monthly incidence of *L. monocytogenes* on farms was 2.8%. Over the last year of the study, a different data collection procedure was used which showed that average monthly prevalence of *L. monocytogenes* in milk obtained from the farms was 7.7%<sup>27</sup>. In a study in Netherlands, six out of 137 sample of raw milk contained *L. monocytogenes*<sup>28</sup>. Another study conducted in a Brazilian milk plant, reported 9.5% of the raw milk samples contaminated with *L. monocytogenes*<sup>29</sup>. In Hungary, 26.1% of the raw milk samples were contaminated with *L. monocytogenes*. Out of the total 29 samples reported to be positive for *L. monocytogenes*, 15 strains belonged to serotype 1/2a, five to 4a, three to 4b and six were 4ab serotype<sup>30</sup>.

Cheese has been reported to be a cause of many Listeriosis outbreaks. Under a survey conducted in England and Wales, samples of milk, cheese and other dairy products were examined over a period of one year. It was reported that *Listeria* strains were most common in soft ripened cow milk cheese. 63 out of 769 samples were reported to carry *L. monocytogenes* and eleven samples of pasteurized milk from four dairies had *L. monocytogenes*. 58% of the *L. monocytogenes* strains were 1/2 serotype and 33% were 4b serotype.

Contamination of soft ripened cheese is mostly restricted to the surface of cheese called the rind. The reason for this has been attributed to the fact that there is a pH gradient formed when the cheese is ripened using bacterial cultures. This increase in pH on the surface of the cheese favors the growth of microorganisms, including *L. monocytogenes*<sup>5, 31</sup>. The ability of *L. monocytogenes* to survive is different in different cheeses. Bacteria are mostly concentrated in the curd with very small number of organisms found in the whey<sup>5</sup>. A study conducted on some European Cheese types also suggested that more frequent *L. monocytogenes* contamination in high moisture cheese than in hard cheese. Same flora was used to ripen soft cheese and hard cheese, but hard cheese showed no Listeria contamination as against the soft cheese that contained the *L. monocytogenes*. Surface cell counts of bacteria were reported to increase even during proper cold storage<sup>31</sup>.

Other milk products like yogurt, cream, ice cream etc also have been reported to be contaminated by *L. monocytogenes*<sup>30</sup>. The bacterial growth has been reported to slow down but not stop in the presence of lactic starter cultures. *L. monocytogenes* has been reported in products like yogurt, buttermilk etc. Acid adaptation has been reported in some *L. monocytogenes* strains, that can help in their survival in acidified foods like yogurt, cottage cheese etc<sup>5, 32</sup>.

#### **2.1.4.2 *Listeria monocytogenes* in meat products**

Meat is another source of *L. monocytogenes* contamination. But it has been suggested that many contaminated meat products contain lesser numbers of organism than the soft cheese. Chicken is reported to be heavily contaminated

with *L. monocytogenes*. Although *L. monocytogenes* does not colonize chickens very easily, younger chickens are more susceptible to colonization. Survival of *L. monocytogenes* on meat is dependent on a number of factors like pH, temperature, type of meat and initial micro flora. Poultry meat is reported to support the growth of this bacterium better than other meats. A pH above 6.0 is more favorable to the *L. monocytogenes*, and a pH of 5.0 or below aids a little growth or no growth at all on meat<sup>5</sup>.

The incoming poultry birds in a poultry processing plant were suggested to be the origin of contamination. But the same genotype was also found on raw poultry products and food contact surfaces which can further act as a source of cross contamination. This suggests the establishment and persistence of some strains in the processing environment. Certain strains can get adapted to their specific niche in the processing environment. The same strain was also found in the processing environment where the meat was cooked to 85 °C. The explanation for this was suggested to be the ability of bacteria to get adapted to certain microenvironments. There could have been an initial contamination from the raw poultry environment, allowing the genotype to get established in the environment<sup>33</sup>. Another study has also suggested that the dependence of final product contamination on the contamination of fresh meat has been overestimated. It was suggested that the poor personal and general hygiene could also be the cause. Workers in food plants and slaughterhouses were reported to carry *Listeria* on their hands and also some of them could be healthy carriers<sup>34</sup>.

There have been outbreaks of Listeriosis due to the consumption of pork in France and other European countries. Pigs harbor *L. monocytogenes* in their intestines. Chilling and cutting in the pork processing plants have been reported to be a major cause of meat contamination, as prevalence of *L. monocytogenes* in chilling and cutting environments has been reported to be 71-100%. Processing environment therefore has been reported to be the primary source of contamination of meat before it is released to the consumers. The serotypes in the processing environments mostly are 1/2a, 1/2b and 1/2c, but serotype 4b has been reported in some French Deli meats<sup>35, 36</sup>.

An outbreak occurred in 24 states of US because of consumption of frankfurters produced at a single facility. The proposed cause of this outbreak was a demolition that was carried out in the plant. Removal of a ceiling refrigeration unit increased the level of contamination in the facility. This consolidates the belief that *L. monocytogenes* form a niche in the production environment and is able to survive there. Although the patients suffering from Listeriosis in this outbreak reported that they heated the frankfurters before eating, either heating was not enough or there was cross-contamination from other foods that came into contact with uncooked frankfurters<sup>37</sup>.

#### **2.1.4.3 *Listeria monocytogenes* in vegetables**

Vegetables have been a source of Listeriosis outbreaks, e.g. the Canada outbreak caused by consumption of contaminated cabbage. In a study conducted on fresh market produce; it was found that radish and potatoes were more frequently contaminated than other vegetables like cauliflower, tomatoes, carrots,

and broccoli. It was suggested that the vegetables that came in contact with soil had more possibility of being contaminated. In case of carrots, a study suggested that some compound in carrot was toxic to *L. monocytogenes*, thus explaining the lack of contamination<sup>38</sup>. *L. monocytogenes* has been reported to be present in fresh market produce as reported by many workers<sup>39, 40, 41, 42</sup>.

A 23-month study conducted in a vegetable processing plant suggested a low incidence of 1.2% *L. monocytogenes* in frozen vegetables. The pathogen was found mainly in green beans and tomatoes. Isolated samples of cauliflower, artichoke and peas were also contaminated. The source of contamination of plant environment was suggested to be contaminated vegetables coming into the plant<sup>43</sup>. Another study conducted on frozen vegetables in Spain also reported a low incidence of 1.8% *L. monocytogenes*. They also suggested that since these products were to be consumed after heat treatment (boiling), so transmission of Listeriosis was very unlikely<sup>44</sup>.

A number of studies have been conducted on the growth and survival of *L. monocytogenes* in vegetables. A study conducted in Cabbage juice suggested that the bacterium was able to survive even in 5% NaCl which is used in brines used in the process of making fermented vegetables. It was also reported that it can survive and grow at a pH of 5.6 or lower. However, the pH of  $\leq 4.8$  was lethal for *L. monocytogenes*<sup>45</sup>.

Some measures have been suggested to reduce the contamination in vegetables. It has been suggested to disinfect the surface of the vegetables before they are cut and processed, also maintaining proper temperature

conditions during processing and storage, preventing the contact of raw vegetables with the finished products have been reported to be helpful. Some other practices like washing the vegetables thoroughly, packing the vegetables in modified atmosphere, or incorporating organic acids and bacteriocins in the packaging material are also found to be effective against *L. monocytogenes*<sup>18</sup>.

#### **2.1.4.4 *Listeria monocytogenes* in seafood**

*Listeria monocytogenes* has been reported to be present in various water bodies like rivers, canals, coastal waters etc. So it is highly probable that the organisms living in these water bodies harbor *L. monocytogenes*. It has been reported by many workers that ready to eat seafood is contaminated with *L. monocytogenes*. Cold smoked salmon and halibut showed an incidence of 34 - 43% and 45 – 60% respectively. The production sites varied in the prevalence of *L. monocytogenes* from <1.4% to 100%, thus suggesting that low prevalence at production sites was possible. In case of heat-treated seafood, *L. monocytogenes* was found in 5 – 12% samples<sup>46</sup>. In Canada, however, the only contaminated seafood found was the one that was imported. The inspection of domestic products showed no *L. monocytogenes* contamination<sup>47</sup>.

Gravad rainbow trout has been found responsible for Listeriosis outbreaks. It is made from raw fillet that is seasoned and then matured by keeping it in the refrigerator for 2 days. It is then packed and stored for 2 – 3 weeks. Seafood has been categorized into different risk categories. High-risk category includes mollusks, raw fish, lightly processed fish products and mildly heat processed products. Low risk seafood includes semi-preserved fish, heat-

processed fish, dry, dried-salted, smoke-dried fish and frozen fish and crustaceans. Although seafood has been found to be contaminated *with L. monocytogenes*, very few outbreaks have been caused with seafood. Reason suggested for this is that seafood-processing plants are usually much smaller than dairy and meat plants, thus sending less number of contaminated products to the market<sup>48</sup>. Some workers suggest raw fish to be the primary source of contamination but some believe that processing environment contaminates the incoming raw material and the end product. There are strong indications that suggest that raw material is not the primary source of contamination of final product. The control options for *L. monocytogenes* have been suggested to improve and carry out proper cleaning and sanitation of the processing plant<sup>49</sup>.

### **2.1.5 Detection methods used in food industry**

Detection of *Listeria monocytogenes* in the food samples is a big challenge faced by the food industry. Countries like USA have a limit of 1 cfu/25 g of the product for this pathogen, which means that the methods used for its identification have to be very sensitive in addition to being fast and reliable. An additional hurdle in their detection is the interference posed by the inhibitory food components. Also *Listeria* is a slow growing pathogen and can easily be outnumbered by other competitors, which makes it hard to culture them<sup>50, 51</sup>.

#### **2.1.5.1 Conventional culture dependent methods**

In food industry conventional plating method has been used until as recently as 2002 for the detection of *L. monocytogenes* and is also used for validation of new technologies. But the conventional methods are time

consuming and it takes around 5 – 6 days to get the results. The most popular protocols used are United States Department of Agriculture – Food Safety and Inspection service (USDA-FSIS) method for meat and poultry products, Federal Drug Administration (FDA) method for dairy, fruits, vegetables and seafood; and Netherlands Government Food Inspection service (NGFIS) method for all the foods. These methods differ in the selective media (Culture medium that suppresses the growth of some microorganisms while allowing the growth of others, this can be done by addition of certain selective agents) that they use. Some workers have expressed doubts about the 100% success of these culture methods<sup>50, 51</sup>.

The culture methods involve three steps – enrichment, isolation and confirmation. The choice of enrichment media is a critical step. Enrichment media increases the relative concentration of desired microorganism in a liquid culture. Since *L. monocytogenes* is a slow growing pathogen, it can easily be outnumbered by the other micro flora present in the sample, so it is required to add bacteriostatic agents like nalidixic acid and acriflavin. The disadvantage of adding these agents is that it makes the recovery of injured *Listeria* very difficult. Also it has been found that diverse foods act in a different way in different media, and it is very important to optimize the media according to the food. So the use of same protocols for different foods has been questioned. Where some foods work better with selective enrichment, others have known to work better with initial non-selective enrichment. In case of non-selective enrichment, the growth medium allows the growth of all the microorganisms without favoring anyone in

particular. Also some bacteriostatic agents used like acriflavin has been reported to affect *L. monocytogenes* more than *L. innocua*, thus leading to an underestimation of *L. monocytogenes*. So a modification has been made in which a chromogenic substance i.e. a substance that changes color, is added in the media, that changes color only in the presence of certain enzymes produced by certain species. This reduces the number of steps for detection of *L. monocytogenes*. Enrichment step has been automated to make it faster and easier. Microplates are also used where a number of cultures can be grown simultaneously and a spectroscopic reader can measure the growth<sup>50, 51</sup>.

#### **2.1.5.2 Immuno-separation**

This method uses beads coated with antibody specific to the pathogen to be detected. The beads are incubated with the homogenized sample to be tested and these beads are then separated either by the use of magnet or by centrifugation. This should concentrate the pathogens thus making them easier to detect without using long enrichment methods. This technique has been tested and has enabled the detection of *L. monocytogenes* within one day by using PCR after immuno-separation, but the sensitivity of this method has been questioned. It was found that only 20% of the added pathogens were recovered using immuno-separation. Also the use of antibodies to differentiate *L. monocytogenes* from other *Listeria* species has met with variable success, because this is done on the basis of virulence factors expressed only by *L. monocytogenes* and expression of virulence factors in vitro has been variable. Vitek Immuno Diagnostic Assay (VIDAS) *Listeria monocytogenes* (VIDAS LMO)

assay is an automated immunoassay system developed by a company called bioMerieux. This assay is an exception as they have been able to target a stable virulence antigen for *L. monocytogenes*<sup>50, 51</sup>.

#### **2.1.5.3 Enzyme Linked Immuno-sorbent Assay (ELISA)**

This method uses an antibody fixed on a microtiter plate that captures the antigen. A secondary antibody carrying an enzyme detects the antigen and a substrate is then added. Substrate gives a color reaction that can be read using an ELISA reader. ELISA kits for this pathogen are commercially available and claim sensitivity similar to culture methods. Test provides results within 30 hrs of receiving the sample<sup>50, 51</sup>.

#### **2.1.5.4 Polymerase Chain reaction (PCR)**

This is a method in which a specific Deoxyribonucleic acid (DNA) fragment is amplified by using a heat stable DNA polymerase and two primers. The ethidium bromide stained amplified fragment can then be detected by agarose gel electrophoresis under Ultra Violet (UV) light. PCR is capable of amplifying small quantities of DNA. But enrichment for 24 – 48 hrs increases the reliability of the test. Another obstacle after the enrichment step is posed by the food components that act as inhibitors of PCR. So sample treatment is required or methods like immuno-separation are used to isolate the pathogens from the inhibitory food matrix. There are PCR kits that are commercially available like BAX PCR system developed by DuPont Qualicon and Probelia assay. The disadvantage of using PCR is that it doesn't differentiate between living and dead bacteria. In order to solve this problem use of Reverse Transcription –

Polymerase Chain Reaction (RT-PCR) has been recommended. This detects the presence of specific Ribonucleic acid (RNA) sequences. When an organism dies, its RNA is degraded quickly as compared to DNA. It uses an enzyme called reverse transcriptase that is capable of creating a complementary DNA from a RNA strand. Oligonucleotide primers are then used to amplify this complementary DNA (cDNA), which is then analyzed the same way as in regular PCR<sup>50, 51</sup>.

#### **2.1.5.5 DNA hybridization**

In this method the presence of a target sequence is detected using a labeled oligo-nucleotide probe of complementary sequence to the target sequence. The labels for identification incorporated into the oligo-nucleotide probe can be radioactive, biotinylated or fluorescent. These tests have been found to be reliable and sensitive. Many commercial testing kits based on this technology are available like GeneTrak DNA hybridization kit. Accuprobe is another test based on the hybridization of labeled DNA probes to virulence factor messenger RNA (mRNA) thus identifying viable cells<sup>50, 51</sup>.

## **2.2 *Salmonellae***

### **2.2.1 Classification**

*Salmonella* is a member of the Family *Enterobacteriaceae*, order Eubacteriales. *Salmonella* nomenclature has been under debate but according to the latest nomenclature used by Center of Disease control (CDC) Genus *Salmonella* is divided into two species; *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into six sub-species designated by names

or Roman numerals. They are; *enterica* or I, *salamae* or II, *arizonae* or IIIa, *diarizonae* or IIIb, *houtenae* or IV and *indica* or VI. *Salmonella bongori* was initially classified as subspecies V but was then determined to be a different species. CDC uses names for serotypes in subspecies I and use antigenic formula for the un-named subspecies. Serotypes are based on the two surface structures; antigen O and H. O antigen is a carbohydrate antigen that is the outermost component of lipopolysaccharide (LPS). H antigen is flagellar antigen ;made up of protein subunits called flagellin. In order to avoid the confusion, serotype names are not italicized and the first letter is capital. 2541 serotypes of *Salmonella* have been identified till 2002 and majority (59%) belongs to *S. enterica* subsp I. In *S. enterica* subsp I, most common O-antigen serogroups are A, B, C1, C2, D and E. 99% of the *Salmonella* infections in humans and warm blooded animals are caused by this group. Serotypes from all the other subspecies and *S. bongori* are usually isolated from cold-blooded animals<sup>52, 53</sup>. It is gram-negative, rod shaped, non-spore forming and facultative anaerobe. Most of the *Salmonella* strains are motile with peritrichous flagella, but some non-motile variants occur occasionally. Most of the strains grow on nutrient agar as smooth colonies, 2 – 4 mm in diameter<sup>54</sup>. Most of the strains are lactose negative i.e. they are not able to use lactose in the growing media but some lactose positive strains have also been reported<sup>55</sup>.

### **2.2.2 Growth conditions**

*Salmonella* is the enteric pathogen that spends a good part of its life in the animal host. The serotypes belonging to *S. enterica* species are mostly

responsible for infections in human beings. The optimum growth temperature for this pathogen ranges from 35 – 37 °C. The minimum temperature required for the growth is 7 °C and the maximum temperature it can tolerate is 49.5 °C. The growth at a temperature < 15 °C is greatly reduced. Some serotypes have been reported to grow at 5.2 °C. The optimum water activity for this pathogen is 0.99, but it can grow in the water activity range of 0.94 to greater than 0.99. pH 7 – 7.5 is the optimum pH for *Salmonella*. It can grow at a pH range of 3.8 – 9.5. But growth in different pH conditions is also affected by other factors like temperature, acidity, nitrite etc. It is capable of growing both in presence and the absence of air. In the presence of Nitrogen, growth is only slightly retarded<sup>56, 57</sup>. Most of the *Salmonella*-caused human gastroenteritis is caused by this pathogen from foods or animal origin like meat and eggs. Fruits and vegetables can also be contaminated by infected manure. Birds and fleas play an important role in the dissemination. So *Salmonella* is capable of colonizing a variety of organisms and has the ability to survive inside or outside of the host<sup>58</sup>. Survival of this pathogen for long periods in refrigerated conditions has also been reported. It can survive in very dry environments like chocolate with a  $a_w$  of 0.3 – 0.5. Exposure to such low water activity might lead to an increase in the heat resistance of the organism. It has also been reported to be less acid resistant at low pH values than the *E.coli*<sup>56, 57</sup>.

### **2.2.3 Distribution**

A global epidemiological survey conducted on Salmonellosis from the year 1990 to 1995 suggested that three serotypes were primarily responsible for the

infection. These serotypes were; Enteritidis, Typhimurium and Typhi. Enteritidis and Typhimurium are the non-typhoidal serotypes and *S. Typhi* causes typhoid fever. *S. Typhi* has its reservoir in humans unlike non-typhoidal serotypes, which infect humans due to the consumption of contaminated food of animal origin. The mean proportion of *Salmonella* isolates that were Enteritidis, increased globally from 25.6% in 1990 to 36.3% in 1995. Most of the Salmonellosis cases due to Enteritidis were caused by consumption of eggs or meat from chickens. Typhimurium that was already reported to be prevalent in Europe and the Americas, was found to be growing in South-East Asia, Western Pacific and African regions. *S. Typhi* incidence was more in the countries where sanitation was an issue. There was a decrease in the proportion of Salmonellosis caused by *S. Typhi* but at the same time the emergence of resistant strains pose a new challenge<sup>59</sup>. Another global survey supported by World Health Organization (WHO) was conducted from 2000 – 2002. *S. Enteritidis* serotype was found to be the most prevalent globally. The incidence of this serotype worldwide was 60% in 2002 as against 36% in 1995. The other two most prevalent serotypes were Typhimurium and Typhi. Other common human serotypes were *S. Agona*, *S. Infantis*, *S. Montevideo*, *S. Saintpaul*, *S. Hadar*, *S. Mbandaka*, *S. Newport*, *S. Thompson*, *S. Heidelberg*, and *S. Virchow*<sup>60</sup>.

### **2.2.3.1 *Salmonella* in animals and birds**

Food borne Salmonellosis is mostly caused by the consumption of animal products. Various farm animals like cows, pigs and poultry have been found to be contaminated with *Salmonella* and in turn contaminate the environment, feed and

the processed end product. Rodents have been reported to harbor *S. Enteritidis* even before it emerged as a major cause of food borne illness. It has been suggested that rodents could have introduced the pathogen in poultry because poultry farms are often inhabited by mice and rats<sup>61</sup>. *S. Dublin* and *S. Typhimurium* have been reported to be the most prevalent serotypes in cattle. *S. Dublin* has been the most prevalent since 1991<sup>62</sup>. Pigs are known to harbor *Salmonella* without showing any symptoms of infection. *S. Choleraesuis* and some types of *S. Typhimurium* are known to cause infection in pigs. 37% of the pig carcasses sampled at the end of slaughter line were found to have *Salmonella*. 19% of the pigs shed *Salmonella* in their faeces and 21% carried it in mesenteric lymph nodes<sup>63</sup>. In another study *Salmonella* was isolated from one or more samples of 47% of the pigs. The highest prevalence of *Salmonella* was in the rectal contents and lowest in the carcasses<sup>64</sup>. *S. Typhimurium* has been reported to be the most prevalent serotype in pigs<sup>63</sup>. Other serotypes commonly found in swine are Panama, London, Infantis, Derby, Brandenburg, and Livingstone<sup>62, 63</sup>. Chicken and Turkey are also known to be contaminated by *Salmonella*. A study conducted in Belgium reported an increase in *Salmonella* incidence on chicken carcasses and other parts during a period of four years. *S. Enteritidis*, *S. Hadar*, *S. Virchow* and *S. Typhimurium* represented the majority of the serotypes isolated from poultry products<sup>65</sup>. In Malaysia, the overall incidence of *Salmonella* in broiler chickens was found to be 38.3%. The broiler carcasses were reported to carry fifteen serovars, the predominant ones were *S. Enteritidis*, *S. Muenchen*, *S. Kentucky* and *S. Blockley*. Incidence of *S. Enteritidis* was

reported to have increased and it was the most prevalent serotype in the processing plant<sup>67</sup>. A study was conducted on the prevalence of *Salmonella* on Turkey while on farm and just before the slaughter. At both the sampling points, the overall prevalence was found to be 33%<sup>66</sup>. A study was also conducted on the wild birds near the broiler chicken houses. Most of the samples consisted of bird droppings found on or near the house. A few intestinal samples and cloacal swabs were also available. Study conducted on four farms at different times suggested that during most of the time periods tested, *Salmonella* spp were found in the samples and thus have the potential to transmit the pathogens to poultry birds<sup>68</sup>.

An outbreak of Salmonellosis occurred among children who visited a reptile exhibit at a zoo. It was reported to be caused by *S. Enteritidis*. Some patients had touched the wooden barrier surrounding the pen of the animal but no one touched the animal. This outbreak suggested the importance of environmental contamination in the transmission of *Salmonella* from reptiles<sup>69</sup>. Salmonellosis associated with exposure to reptiles has been reported quite often. Lizards have been reported to be asymptomatic carriers i.e. they do not show any symptoms of infection of *Salmonella* spp. Iguana, a very popular pet reptile has been reported to carry *S. Marina*. Most of the patients reported were infants. Direct contact with the reptile iguana was not necessary, but environmental contamination and contact with others who were in direct contact with the animal were the suggested means<sup>70</sup>. Four cases of Salmonellosis due to the serotype *S. Pomona* were reported in Wisconsin in the year 2004. All the four patients

were found to be in contact with small turtles bought from the souvenir shops at tourist locations. In Wyoming, two cases of Salmonellosis caused by *S. Typhimurium* were reported and the patients were reported to have been in contact with pet turtles<sup>71</sup>. Human Salmonellosis caused by *S. Thompson* has also been reported caused by exposure to animal-derived pet treats of beef and seafood origin<sup>72</sup>.

#### **2.2.3.2 *Salmonella* in Water and Soil**

*Salmonella* has been reported to be present in different kinds of water bodies like fresh water, coastal water, ground water and even the sewage. Human and animal faeces are considered to be a major cause of environmental contamination. Its survival in the environment during its transmission shows its resistance to the environmental factors. Its survival in water is neither seasonal nor dependent on the water temperature. It has been reported to survive in the sewage waste for 10-15 days in spite of the measures taken to sanitize the sewage. A study conducted on the natural aquatic systems revealed the presence of 41 serotypes that were identified. Thirteen isolates were characterized by an incomplete serotype. Thirty-five different serotypes were found in the river water samples and diversity of serotypes was more during floods. *S. Typhimurium* was the most dominant serotype. In the wastewater samples, only 14 different serotypes were identified and *S. Newport* was the predominant serotype<sup>58, 73</sup>.

*Salmonella* is also reported to be present in soil and has the ability to grow and survive in the soil environment. Soil can be contaminated due to the contact

with water from underground springs, rain run off etc. Soil provides the bacteria with nutrients due to release of organic molecules from the algal cells. It can survive and grow in soil for one year<sup>58</sup>.

## **2.2.4 *Salmonella* as food-borne pathogen**

### **2.2.4.1 *Salmonella* in meat**

Apart from eggs, poultry meat also acts as source of *Salmonella*. Various studies conducted in different countries show incidence of this pathogen in poultry meat and meat products. There was an increase in Salmonellosis involving *Salmonella* Group D cases in Massachusetts during June and July of 1996. It was found out that these illnesses were linked to a single restaurant, and the serotype *S. Javiana* was isolated from the clinical samples and a left over chicken sandwich. The suggested route of the pathogen was from food handlers to the food<sup>74</sup>. In Belgium, a study suggested greater contamination of chicken meat with *Salmonella* than the turkey meat. Twenty-six serotypes were isolated from poultry products during a period from June 1994 to December 1996. The predominant serotypes were; *S. Enteritidis*, *S. Hadar* and *S. Virchow*. *S. Typhimurium*, *S. Newport* and *S. Infantis* were also frequently isolated<sup>65</sup>. In Portugal, 60% of the poultry products tested were contaminated with *Salmonella*. *S. Enteritidis* was the most predominant followed by *S. Hadar* and *S. Virchow*. Four turkey samples tested gave four serotypes of *Salmonella*<sup>75</sup>. During a three-year study in Albania, 6.5% of the chicken meat samples were contaminated with *Salmonella* and *S. Enteritidis* was the predominant serotype isolated<sup>76</sup>. In Thailand, the prevalence of *Salmonella* in chicken meat at the market was

57%<sup>77</sup>. A three-year study was carried out in Ireland testing all kinds of meats for *Salmonella*. The recovery was highest for poultry meat, 3.1% for chicken and 2.8% for turkey meat. Recovery from porcine meat was 2.1% and that from ovine and bovine meat was 0.2 and 0.16% respectively. *S. Typhimurium* was the most predominant serotype, and *S. Kentucky* and *S. Derby* were also frequently isolated<sup>78</sup>.

In 2004, a multi-state Salmonellosis outbreak occurred in USA due to the consumption of contaminated ground beef. *S. Typhimurium* caused the outbreak through ground beef that was eaten raw or was undercooked. The strain of *Salmonella* was multi- drug resistant Phage type DT 104<sup>79</sup>. Thailand reported that 29% of the pig meat available in the market was contaminated. 3% of the dairy cows were also carrying *Salmonella*<sup>77</sup>.

#### **2.2.4.2 *Salmonella* in eggs**

*S. Enteritidis* incidence started increasing as early as the mid1970s and by 1990 it displaced *S. Typhimurium* as the primary cause of Salmonellosis. *S. Enteritidis* is the only human pathogen that routinely contaminates eggs and eggs have been the main cause of *S. Enteritidis* outbreaks. It has been suggested that stringent measures taken by many countries to control *S. Pullorum* incidence in poultry lead to its replacement with *S. Enteritidis*. *S. Enteritidis* does not produce any symptoms in the chickens. Contamination in eggs usually occurs before the shell deposition, and thus gains entry to egg yolk or albumen through the reproductive tract. Egg environment is not very hospitable for *S. Enteritidis* because of the presence of lysozyme and lack of iron

in the albumen and presence of antibodies in the yolk. But some variants are adapted to the egg environment and can survive in it<sup>80</sup>. The survival of *S. Enteritidis* in yolk is more likely than its survival in the albumen. But if the eggs are stored at room temperature for a long time, bacteriostatic agents in albumen can not destroy all *S. Enteritidis* cells and these cells can migrate to egg yolk and survive better than in the albumen<sup>81</sup>. It has been estimated that in USA 1 in 12000 eggs is *S. Enteritidis* contaminated. In Great Britain, *Salmonella* spp incidence is one in 15000 eggs. There is greater chance of *S. Enteritidis* infection by consumption of egg products like Mayonnaise, cream, ice cream etc that have not undergone heat treatment<sup>82, 83</sup>.

In 2003, *S. Typhimurium* outbreak occurred in Oregon due to the consumption of egg salad. The reason of egg salad contamination could be improper cooking of eggs, inadequate cooling of eggs or improper handling by the employees<sup>84</sup>.

#### **2.2.4.3 *Salmonella* in fruits and vegetables**

The incidence of human pathogens on fresh produce is a major cause of food borne illness. There has been Salmonellosis outbreaks associated with the consumption of tomatoes, lettuce, cantaloupes, un-pasteurized orange juice etc. Contamination of fresh produce can occur from a variety of sources like water, soil, humans, contact with raw meat and animals. Cut fruits kept at ambient temperature can encourage the growth of *Salmonella* to large numbers. Lettuce and tomatoes have been the source of Salmonellosis outbreaks in the US. The serotype associated with tomato outbreak was *S. Baildon*. In 2004 other

outbreaks due to roma tomatoes were reported in US and Canada. These cases yielded *Salmonella* serotypes Javiana, Typhimurium, Anatum, Thompson, Muenchen, and Group D untypable. In another outbreaks associated with Roma tomatoes, *S. Braenderup* was isolated. In Canada, the serotype found responsible for the outbreak was *S. Javiana*. In Florida, an outbreak occurred due to the consumption of un-pasteurized orange juice. *S. Hartford* and *S. enterica* serotype Gaminara were isolated from stool samples of one patient<sup>85, 86</sup>.

Measures suggested to reduce *Salmonella* incidence on produce include washing the fresh produce thoroughly before consumption. Some people consumed cantaloupes without washing then assuming they had already been washed or because they did not consume the rind. But if the outer layer is contaminated with bacteria, these bacteria can gain entry into the fruits because of cutting. In the 1999 US outbreak due to consumption of tomatoes *S. Baildon* was found to be responsible. A study was carried out to see if chlorinated water was able to help in reducing the serotype on tomatoes and lettuce. The *Salmonella* serotype was reported to possess unusual resistance to acid pH. They concluded that it was difficult to get rid of this bacteria using chlorine at a concentration of 200 µg/ml. So it was suggested that measures should be taken to improve agronomic practices, processing, distribution and storage<sup>87, 88</sup>.

#### **2.2.4.4 *Salmonella* in dairy products**

Dairy products especially cheese has been reported to be the cause of many Salmonellosis outbreaks. Most of the times, faulty pasteurization or contamination after pasteurization has been reported to be the cause. In 1989,

an outbreak occurred in Wisconsin and Minnesota due to the consumption of Mozzarella cheese. *Salmonella choleraesuis* subsp. *choleraesuis* serotype javiana was the serotype responsible for the outbreak. Acid adaptation was found to be an important survival mechanism of *Salmonella* spp. in fermented dairy products<sup>89</sup>. A major Canada-wide outbreak of gastroenteritis due to *Salmonella enterica* serotype Enteritidis phage type (PT) 8 was reported in 1998. The cause was contaminated cheese in a commercial lunch pack product<sup>90</sup>. In 1994, an outbreak occurred in Ontario due to consumption of an unpasteurized soft cheese. The serotype responsible for the outbreak was *S. Berta*. The cheese was sold in Farmers market and was prepared on the farm. The subtyping results suggested that cheese was contaminated from the chicken carcasses<sup>91</sup>. In February 1997 *S. Typhimurium* var Copenhagen caused Salmonellosis outbreak due to unpasteurized Mexican-style cheese. Another outbreak peaked in April 1997 and was caused by a non-Copenhagen variant of *S. Typhimurium*. During this outbreak, *S. Typhimurium* was isolated from 79 persons who ate fresh Mexican-style cheese from street vendors and from cheese samples and raw milk<sup>92</sup>.

#### **2.2.4.5 *Salmonella* in seafood products**

*Salmonella* is capable of surviving in water bodies and thus there is a good chance of the prevalence of these bacteria in aquatic animals. *Salmonella* has been reported in fish and other fishery products in many Asian countries. A study conducted in India reported 30% of finfish, 20% of clams and 5% of Shrimp tested to be contaminated with *Salmonella* spp<sup>93</sup>. There is possibility of seafood

to be contaminated if it is farmed in polluted water. Fish from clear open water can get contaminated after harvesting. There are many incidences of contaminated shrimp grown in poor quality water<sup>94</sup>. A study conducted by US FDA reported an incidence of 7.2% and 1.3% in imported and domestic seafood respectively. The most frequent serotypes in import seafood were *S. Weltevreden*, *S. Senftenberg*, *S. Lexington*, and *S. Paratyphi-B*. The top 20 serotypes included *S. Enteritidis*, *S. Newport*, *S. Thompson*, *S. Typhimurium* and *S. Anatum*<sup>95</sup>. A study conducted in US on the incidence of *Salmonella* spp. in Oysters, suggested that the prevalence was bay specific and also related to weather. 7.4% of all the oysters tested were positive for *Salmonella*. There were 13.4% oysters positive for *Salmonella* in summers as against 1.6% in winters. *S. Newport* was the predominant serotype found in oysters<sup>96</sup>.

## **2.2.5 Detection methods used in food industry**

### **2.2.5.1 Culture dependent methods**

Culture dependent methods are usually reliable but are more time consuming. Sometimes, *Salmonella* detection using culture methods can take from 5 to 7 days. United States Department of Agriculture (USDA) and United States Food and Drug Administration (FDA), recommends biochemical and serological tests in addition to the culture methods for the identification of *Salmonella* spp. The culture methods are labor extensive and involve a tedious procedure. The steps include pre-enrichment in a non-selective broth that allows all the microorganisms to grow without favoring any of them specifically, followed by transfer to enrichment broth. Both FDA and USDA methods recommend

Rappaport-Vassiliadis (RV) medium and tetrathionate (TT) broth for selective enrichment. They favor the growth of *Salmonella* over other microorganisms. The enriched samples are then plated on selective agar plates to further select only the *Salmonella* bacteria. FDA recommends Bismuth Sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar while USDA recommends Brilliant Green Sulfa agar (BGS), Xylose lysine Tergitol™ 4 agar (XLT4) or Double modified lysine iron agar (DMLIA). The colonies that are typical of *Salmonella* in color and morphology, are then tested using screening media. Triple sugar iron agar (TSI) and Lysine iron agar (LIA) are used for screening the colonies from the selective agar plates to make sure they are *Salmonella*. The suspected *Salmonella* samples can then be verified using biochemical and serological tests. USDA recommends using commercial kits like VITEK developed by bioMeriux or traditional methods for biochemical tests<sup>97, 98</sup>.

#### **2.2.5.2 Immunoassays**

A number of antibody-based assays for the detection of *Salmonella* spp in food are available commercially. These methods are based on Enzyme Linked ImmunoSorbent Assay (ELISA), Latex agglutination (LA), Immuno-precipitation (Ab-ppt) etc. These methods need to be validated before they can be used in food for the analysis. Immuno-magnetic separation (IMS) after pre-enrichment can be used to remove *Salmonella* cells from the pre-enrichment media, thus expediting the procedure because secondary enrichment in which usually selective agents are used might not be needed. A comparative study conducted to see the efficiency of IMS suggested that it could be used as an alternative to

secondary enrichment. But in order to increase the chance of *Salmonella* recovery, both IMS and secondary enrichment might be needed<sup>98, 99</sup>. The detection time can be reduced from the 5-7 days taken by the culture methods to time ranging from a few hours to 1 –2 days.

In ELISA, antibodies specific to *Salmonella* are used to detect the bacteria. Either the antibody or the antigen can be coated on the micro-titer plates. The reaction of the antibody or the antigen coated on the plate with the antigen or the antibody respectively added onto it can be measured by a color reaction or fluorescence. This reaction occurs because of an enzyme linked secondary antibody that gives a reaction with the substrate. If the antibody is able to recognize the antigen, there would be a reaction that can be read by the ELISA reader.

In case of Latex agglutination (LA), an antibody or the antigen can be coated on the latex beads. When a sample containing the antigen or the antibody specific to the antibody or the antigen coated on the latex, is added to the latex, a visible agglutination will occur. Degree of agglutination can then be measured using automated techniques<sup>100</sup>.

A number of modifications have been done to these methods and new methods introduced based on analysis using antibodies.

#### **2.2.5.3 DNA based methods**

A number of commercially available kits use Deoxyribonucleic acid (DNA)-based assays for *Salmonella* detection. They are usually based on Polymerase Chain Reaction (PCR) or DNA-DNA hybridization. PCR is based on the use of

specific oligonucleotides called primers that are able to recognize a particular sequence on the bacterial DNA. Using a DNA polymerase and nucleotides with other reaction components in a thermocycler, a very small amount of DNA can be amplified to large amounts. The amplified DNA can then be analyzed on the gel using electrophoresis. Many modifications of this technique are being tested for their potential to be used in the food industry. Immuno-magnetic separation used with PCR can help in decreasing the assay time.

Another strategy adopted based on the bacterial DNA is the use of DNA probes. These labeled DNA probes are able to hybridize to the specific sequences on the *Salmonella* DNA. The probes can be radioactive, biotinylated or fluorescent. Commercial kits based on probes are also available for *Salmonella* detection<sup>101</sup>.

### **2.3 Biosensor**

The purpose of testing these antibodies was to use the selected antibodies in biosensors to detect *L. monocytogenes* and *Salmonella* in food. The proposed biosensor would be based on CANARY™ Technology. Based on this technology, an engineered biosensor cell line would be developed consisting of;

- Membrane bound pathogen specific antibodies
- Calcium sensitive bioluminescent molecule

The cross-linking of specific pathogens to the antibodies on the biosensor triggers the release of intracellular Calcium. This Calcium in turn activates the

luminescent properties of the bioluminescent protein. The luminescence can be measured using a luminometer<sup>106</sup>.

### **III MATERIALS AND METHODS**

#### **3.1 Identification and procurement of monoclonal antibodies**

Both academic and commercial sources of monoclonal antibodies were explored in order to serve our purpose of finding the monoclonal antibodies specific to *L. monocytogenes* and *Salmonella* spp. These antibodies were to be used in the biosensors for detection of *L. monocytogenes* and *Salmonella* spp. respectively in food.

For *L. monocytogenes*, eight monoclonal antibodies were procured. Five of them were from commercial sources namely Abcam and Biodesign. Three antibodies were procured from research labs; two from Dr Arun K. Bhunia, Professor, Department of Food Science, Purdue University and one from Dr A Jerald Ainsworth, Professor, College of veterinary medicine, Mississippi State University. All the antibodies except for the one from Dr Ainsworth's lab recognized the cell surface proteins. Dr Ainsworth's antibody was specific to Listeriolysin (LLO), a toxin secreted by *L. monocytogenes*. Information about these antibodies is summarized in Table 1.

Table 1 Monoclonal antibodies specific to *Listeria monocytogenes* procured from different sources

<b>Source</b>	<b>Clone</b>	<b>Isotype</b>	<b>Target</b>	<b>Specificity</b>
Bio Design	LZH1	IgG <sub>1</sub>	Outer membrane fraction and intact cells of <i>L. monocytogenes</i>	<i>L. monocytogenes</i>
Bio Design	LZF7	IgG <sub>2a</sub>	Outer membrane fraction and intact cells of <i>L. monocytogenes</i>	<i>L. monocytogenes</i>
Abcam	LZH1	IgG <sub>1</sub>	Outer membrane fraction and intact cells of <i>L. monocytogenes</i>	<i>L. monocytogenes</i>
Abcam	LZF7	IgG <sub>2a</sub>	Outer membrane fraction and intact cells of <i>L. monocytogenes</i>	<i>L. monocytogenes</i>
Abcam	LZA2	IgM	Outer membrane fraction and intact cells of <i>L. monocytogenes</i>	<i>L. monocytogenes</i>
Dr Bhunia	EM7G1	IgG1	Cell surface proteins	<i>L. monocytogenes</i>
Dr Bhunia	C11E9	IgG2b	Cell surface proteins	<i>L. monocytogenes</i> and <i>L. innocua</i>
Dr Ainsworth	SE8		Listeriolysin (LLO)	<i>L. monocytogenes</i>

For *Salmonella*, seven monoclonal antibodies were procured from the sources namely; Abcam, Bio Design, US Biological and Gene Tex. All these antibodies were supposed to identify all *Salmonella* serovars. Information about these antibodies is summarized in Table 2.

Table 2 Monoclonal antibodies specific to *Salmonella* procured from different sources

<b>Source</b>	<b>Clone</b>	<b>Isotype</b>	<b>Immunogen</b>	<b>Cellular localization</b>	<b>Specificity</b>
Abcam	6321	IgG	Tissue/cell preparation	Whole cell	<i>Salmonella</i> species
GeneTex	6321	IgG	Tissue/cell preparation	Whole cell	<i>Salmonella</i> species
GeneTex	6361	IgG2a	Tissue/cell preparation	Whole cell	<i>Salmonella</i> species
USBiological	1.B.484	IgG	Crude flagellar preparation from <i>Salmonella</i> sp.	Whole cell	<i>Salmonella</i> species
BioDesign	BID32	IgG	Crude flagellar preparation	Whole cell ELISA	<i>Salmonella</i> species
BioDesign	6301	IgG1	Tissue/cell preparation	Whole cell	Most <i>Salmonella</i> Sp
BioDesign	IFR0111	IgG2b	Flagellar preparation from <i>S. hadar</i>		Broad reactivity to <i>Salmonella</i>

To test these monoclonal antibodies for specificity and sensitivity, they were analyzed using **Enzyme-Linked ImmunoSorbent Assay (ELISA)**.

### **3.2 Enzyme-Linked ImmunoSorbent Assay (ELISA)**

**Enzyme-Linked ImmunoSorbent Assay (ELISA)** was carried out using procedure described by Geng et al with slight modifications<sup>103</sup>.

#### Preparation of bacterial cultures

The bacterial cultures to be used for the analysis of monoclonal antibodies were grown overnight in broth. Different broths were used for different bacteria used in the study. Luria Bertani Broth (LB) from Sigma-Aldrich was used to grow all the gram-negative bacteria; *Salmonella*, *E.coli*, *Vibrio*, *Shigella*, *Proteus*, *Citrobacter* and *Enterobacter*. Brain Heart Infusion (BHI) from Difco was used to grow all gram-positive bacteria; *Listeria*, *Enterococcus*, *Staphylococcus* and *Bacillus* except for the Lactic acid bacteria. In case of the Lactic acid bacteria; for *Lactobacilli*, MRS broth developed by de Man, Rogosa and Sharpe procured from Difco was used and M17 from Difco was used for *Streptococci*. All these bacteria except for *Bacilli* were grown in their respective media at 37 °C. *Bacillus* strains were grown at 30 °C.

#### Coating of plates

Bacterial cells from overnight cultures were harvested by centrifugation (6000xg, 4 °C and 10 min). Cells were washed once using 0.05M carbonate coating buffer containing 0.15M Sodium Carbonate and 0.35M Sodium Bicarbonate, pH 9.6. Cells were suspended in the carbonate buffer again and adjusted for the required turbidity value. 50 µl of the bacterial suspension in

carbonate buffer was used to coat each well of 96 well flat-bottomed microtiter plates (Nunc 96 well Immunoassay plates from Fisher Scientific) and incubated at 4°C for 12-14 hrs.

#### Washing the plates

Microplate washer was used to wash the plates. The washing buffer used was Phosphate Buffered Saline (PBS) containing 0.2M Phosphate and 1.5M NaCl with a pH of 7.4 and 0.05% tween added to it (PBS-T). The plates were washed four times in a microplate washer using PBS-T.

#### Addition of primary antibody

Primary antibody that was the monoclonal antibody to be tested was diluted using 5% non-fat dry milk in PBS, which acts as a blocking buffer. Blocking buffer contains a protein unrelated to the one under consideration. In this case the unrelated protein was the milk protein that is not related to the bacterial surface antigen being tested, that binds at the spaces left empty by the antigen. Thus the blocking buffer prevents the occurrence of false results. 200µl of the desired dilution of primary antibody to be tested was added to the wells. The plates were covered with plastic and incubated at room temperature for 2h.

#### Washing

The primary antibody was removed from the plates and plates were washed four times with PBS-T using the microplate washer

#### Addition of secondary antibody

100 µl of secondary anti-species antibody conjugated to Horseradish peroxidase, diluted to a concentration of 400 ng ml<sup>-1</sup> in blocking buffer (5% non-

fat dry milk in PBS) immediately before use was added to each well. The plates were again covered with plastic and incubated for 1 h at room temperature.

#### Washing

The plates were washed four times with PBS-T using a microplate washer.

#### Addition of substrate

100 µl of the 3, 3', 5, 5'- Tetramethylbenzidine (TMB) Liquid substrate solution from Sigma per well was added using a multi-channel pipette. The plates were incubated for 1h in a dark area at room temperature. After 1 h, 100µl stop solution for TMB substrate from Sigma that changes the color from Blue to Yellow so that it can be read at 450nm was added to stop the reaction and absorbance was measured at 450nm.

### **3.3 Testing optimum antibody and antigen concentration**

For both *L. monocytogenes* and *Salmonella*, one strain of each bacterium was selected and used for preliminary study of the monoclonal antibodies specific to *L. monocytogenes* or *Salmonella* and also to optimize the antigen concentration to be used in the study. Different concentrations of the overnight cultures of these strains were coated on the ELISA plates and the concentration that gave satisfactory results was chosen. Also different concentrations of the monoclonal antibodies were used at each antigen concentration to see the antibody concentration that gave a good signal.

### 3.4 Testing reactivity of respective antibodies with *L. monocytogenes* and *Salmonella* strains

For testing the monoclonal antibodies specific to *L. monocytogenes*, eight different strains of *L. monocytogenes* were coated on ELISA Microtiter plates. For *Salmonella*, sixty different strains were coated on the plates and monoclonal antibodies specific to *Salmonella* spp were tested for their reactivity to these strains. The reactivity of monoclonal antibodies to these strains was recorded based on the ELISA reading. The bacterial strains used to determine the reactivity of monoclonal antibodies specific to *L. monocytogenes* and *Salmonella* antibodies are listed in Table3 and Table 4 respectively.

Table 3 *Listeria monocytogenes* strains used for testing monoclonal antibodies specific to *Listeria monocytogenes*

Code	Genus	Species	Serotype
ATCC15313	<i>Listeria</i>	<i>Monocytogenes</i>	1/2a
ATCC19114	<i>Listeria</i>	<i>Monocytogenes</i>	4a
ATCC13932	<i>Listeria</i>	<i>Monocytogenes</i>	4b
ScottA	<i>Listeria</i>	<i>Monocytogenes</i>	
ASM	<i>Listeria</i>	<i>Monocytogenes</i>	2
ATCC19112	<i>Listeria</i>	<i>Monocytogenes</i>	
497	<i>Listeria</i>	<i>Monocytogenes</i>	
478	<i>Listeria</i>	<i>Monocytogenes</i>	

Table 4 *Salmonella* spp strains used for testing monoclonal antibodies specific to *Salmonella*

<b>Code</b>	<b>Genus</b>	<b>Serotype</b>
349	<i>Salmonella</i>	Typhi
196	<i>Salmonella</i>	Typhimurium
370	<i>Salmonella</i>	Typhimurium
373	<i>Salmonella</i>	Typhimurium
23	<i>Salmonella</i>	Typhimurium
64	<i>Salmonella</i>	Typhimurium
66	<i>Salmonella</i>	Typhimurium
86	<i>Salmonella</i>	Typhimurium
96	<i>Salmonella</i>	Typhimurium
122	<i>Salmonella</i>	Typhimurium
163	<i>Salmonella</i>	Typhimurium
21544	<i>Salmonella</i>	Newport
17619	<i>Salmonella</i>	Newport
21532	<i>Salmonella</i>	Newport
352	<i>Salmonella</i>	Group B
353	<i>Salmonella</i>	Group B
354	<i>Salmonella</i>	Group B
197	<i>Salmonella</i>	Enteritidis
522	<i>Salmonella</i>	Enteritidis
520	<i>Salmonella</i>	Kentucky
3	<i>Salmonella</i>	Kentucky
4	<i>Salmonella</i>	Kentucky
7	<i>Salmonella</i>	Kentucky
11	<i>Salmonella</i>	Kentucky
12	<i>Salmonella</i>	Kentucky
62	<i>Salmonella</i>	Kentucky
73	<i>Salmonella</i>	Kentucky
89	<i>Salmonella</i>	Kentucky
108	<i>Salmonella</i>	Kentucky
157	<i>Salmonella</i>	Kentucky
216	<i>Salmonella</i>	Kentucky
232	<i>Salmonella</i>	Kentucky
244	<i>Salmonella</i>	Kentucky
514	<i>Salmonella</i>	Schwarzenberg
513	<i>Salmonella</i>	Heidelberg
530	<i>Salmonella</i>	Heidelberg
531	<i>Salmonella</i>	Heidelberg
83	<i>Salmonella</i>	Heidelberg
111	<i>Salmonella</i>	Heidelberg

Table 4 Contd.

<b>Code</b>	<b>Genus</b>	<b>Serotype</b>
112	<i>Salmonella</i>	<i>Heidelberg</i>
184	<i>Salmonella</i>	<i>Heidelberg</i>
185	<i>Salmonella</i>	<i>Heidelberg</i>
518	<i>Salmonella</i>	<i>Seftenberg</i>
519	<i>Salmonella</i>	<i>Infantis</i>
517	<i>Salmonella</i>	<i>Infantis</i>
524	<i>Salmonella</i>	<i>Infantis</i>
515	<i>Salmonella</i>	<i>Thompson</i>
521	<i>Salmonella</i>	<i>Reading</i>
523	<i>Salmonella</i>	<i>Menhaden</i>
525	<i>Salmonella</i>	
526	<i>Salmonella</i>	<i>Montevideo</i>
196	<i>Salmonella</i>	<i>Montevideo</i>
528	<i>Salmonella</i>	<i>Amager</i>
529	<i>Salmonella</i>	<i>Taxomy</i>
18	<i>Salmonella</i>	<i>Istanbul</i>
30	<i>Salmonella</i>	<i>Ohio</i>
250	<i>Salmonella</i>	<i>Agona</i>
251	<i>Salmonella</i>	<i>Agona</i>
357	<i>Salmonella</i>	
362	<i>Salmonella</i>	

### 3.5 Testing cross-reactivity of antibodies with other bacteria

Cross-reactivity of the antibodies to other bacterial species and also to bacteria from other genus was carried out to determine the specificity of these monoclonal antibodies. In case of monoclonal antibodies specific to *L. monocytogenes*, other *Listeria* species were used. Cross-reactivity of these antibodies was tested against 10 strains each of *E.coli* and *Salmonella*. Some gram-positive organisms were also tested. The bacterial strains used to determine the cross-reactivity of monoclonal antibodies specific to *L. monocytogenes* are listed in Table 5, 6, 7 and 8.

Monoclonal antibodies for *Salmonella* were test against 10 *E.coli* strains, some other gram-negative bacteria and some gram-positive bacteria. Bacterial strains used to test the *Salmonella* monoclonal antibodies are listed in Tables 9, 10 and 11.

Table 5 List of other *Listeria* species that were used to test the cross-reactivity of monoclonal antibodies specific to *Listeria monocytogenes*.

<b>Code</b>	<b>Genus</b>	<b>Species</b>
ATCC33090	<i>Listeria</i>	<i>Innocua</i>
ATCC19119	<i>Listeria</i>	<i>Ivanovii</i>
ATCC19120	<i>Listeria</i>	<i>Grayi</i>
ATCC35897	<i>Listeria</i>	<i>Welshineri</i>
494	<i>Listeria</i>	<i>Welshineri</i>
503	<i>Listeria</i>	<i>Ivanovii</i>
491	<i>Listeria</i>	<i>Innocua</i>
495	<i>Listeria</i>	<i>Welshineri</i>
488	<i>Listeria</i>	<i>seeligeri</i>
487	<i>Listeria</i>	<i>innocua</i>
507	<i>Listeria</i>	<i>ivanovii</i>
501	<i>Listeria</i>	<i>seeligeri</i>

Table 6 List of gram positive bacteria tested for their reactivity to monoclonal antibodies specific to *L. monocytogenes*

<b>Code</b>	<b>Genus</b>	<b>Species</b>
TB1339	<i>Pediococcus</i>	<i>Cerevisae</i>
8292	<i>Lactococcus</i>	<i>Crescentoids</i>
ATCC19433	<i>Streptococcus</i>	<i>Faecalis</i>
Y9SL1	<i>Streptococcus</i>	<i>Thermophilus</i>
Y2SL1	<i>Streptococcus</i>	<i>Thermophilus</i>
Y5SL2	<i>Streptococcus</i>	<i>Thermophilus</i>
Y3LB2	<i>Lactobacillus</i>	<i>Acidophilus</i>
Y2LB2	<i>Lactobacillus</i>	<i>Acidophilus</i>
ATCC367	<i>Lactobacillus</i>	<i>Brevis</i>
NCD0352	<i>Lactobacillus</i>	<i>Plantarum</i>
OGIRF –M	<i>Enterococcus</i>	<i>Faecalis</i>
OGIRF –P	<i>Enterococcus</i>	<i>Faecalis</i>
UV202	<i>Enterococcus</i>	<i>Faecalis</i>
JH2-2	<i>Enterococcus</i>	<i>Faecalis</i>
OGIRF	<i>Enterococcus</i>	<i>Faecalis</i>
CVM99	<i>Enterococcus</i>	<i>Faecalis</i>
CVM15	<i>Enterococcus</i>	<i>Faecalis</i>
CVM14	<i>Staphylococcus</i>	<i>Aureus</i>
BR151	<i>Bacillus</i>	<i>Subtilis</i>
ATCC13061	<i>Bacillus</i>	<i>Cereus</i>
Y1LB1	<i>Lactobacillus</i>	<i>Bulgaricus</i>
Y5LB1	<i>Lactobacillus</i>	<i>Bulgaricus</i>
Y9LB1	<i>Lactobacillus</i>	<i>Bulgaricus</i>
Y12LB1	<i>Lactobacillus</i>	<i>Bulgaricus</i>
Y4LB2	<i>Lactobacillus</i>	<i>Acidophilus</i>
Y14LB2	<i>Lactobacillus</i>	<i>Acidophilus</i>
C3LB2	<i>Lactobacillus</i>	<i>Acidophilus</i>
Y10SL1	<i>Streptococcus</i>	<i>Thermophilus</i>
Y13SL1	<i>Streptococcus</i>	<i>Thermophilus</i>
Y14SL1	<i>Streptococcus</i>	<i>Cremoris</i>
C3SL2	<i>Streptococcus</i>	<i>Cremoris</i>
H2Staph	<i>Staphylococcus</i>	
C5G	<i>Bacillus</i>	
C7G	<i>Bacillus</i>	

Table 7 List of *E.coli* strains used to test *L. monocytogenes* monoclonal antibodies for their cross-reactivity

<b>Code</b>	<b>Genus</b>	<b>Species</b>	<b>Serotype</b>
157	<i>Escherichia</i>	<i>Coli</i>	O78:H11
245	<i>Escherichia</i>	<i>Coli</i>	O1:K1:H7
247	<i>Escherichia</i>	<i>Coli</i>	O127:NM
141	<i>Escherichia</i>	<i>Coli</i>	O26:H11
253	<i>Escherichia</i>	<i>Coli</i>	O167:H5
246	<i>Escherichia</i>	<i>Coli</i>	O3:K2ab:H2
166	<i>Escherichia</i>	<i>Coli</i>	O111:H11
261	<i>Escherichia</i>	<i>Coli</i>	O128:H2
133	<i>Escherichia</i>	<i>Coli</i>	O2:K1:H7
85	<i>Escherichia</i>	<i>Coli</i>	O157:H7

Table 8 List of *Salmonella* strains to test *L. monocytogenes* monoclonal antibodies for their cross-reactivity

<b>Code</b>	<b>Genus</b>	<b>Serotype</b>
349	<i>Salmonella</i>	Typhi
196	<i>Salmonella</i>	Typhimurium
21544	<i>Salmonella</i>	Newport
17619	<i>Salmonella</i>	Newport
21532	<i>Salmonella</i>	Newport
520	<i>Salmonella</i>	Kentucky
352	<i>Salmonella</i>	Group B
514	<i>Salmonella</i>	Schwarzenberg
197	<i>Salmonella</i>	Enteritidis
513	<i>Salmonella</i>	Heidelberg

Table 9 List of *E.coli* strains used to test *Salmonella* monoclonal antibodies for their cross-reactivity

<b>Code</b>	<b>Genus</b>	<b>Species</b>	<b>Serotype</b>
157		<i>Coli</i>	078:H11
245	<i>Escherichia</i>	<i>Coli</i>	O1:K1:H7
247	<i>Escherichia</i>	<i>Coli</i>	O127:NM
141	<i>Escherichia</i>	<i>Coli</i>	O26:H11
253	<i>Escherichia</i>	<i>Coli</i>	O167:H5
246	<i>Escherichia</i>	<i>Coli</i>	O3:K2ab:H2
166	<i>Escherichia</i>	<i>Coli</i>	O111:H11
261	<i>Escherichia</i>	<i>Coli</i>	O128:H2
133	<i>Escherichia</i>	<i>Coli</i>	O2:K1:H7
85	<i>Escherichia</i>	<i>Coli</i>	O157:H7

Table 10 List of gram-negative bacteria used to test the *Salmonella* monoclonal antibodies for their cross-reactivity

<b>Code</b>	<b>Genus</b>	<b>Species</b>
BB120	<i>Vibrio</i>	<i>Harveyi</i>
ATCC12022	<i>Shigella</i>	<i>Flexneri</i>
ATCC25931	<i>Shigella</i>	<i>Sonnei</i>
ATCC13315	<i>Proteus</i>	<i>Vulgaris</i>
ATCC8090	<i>Citrobacter</i>	<i>Freundii</i>
ATCC13048	<i>Enterobacter</i>	<i>Aerogenes</i>

Table 11 List of gram-positive bacteria used to test the *Salmonella* monoclonal antibodies for their cross-reactivity

Code	Genus	Species
Y9LB1	<i>Lactobacillus</i>	<i>bulgaricus</i>
NCD0352	<i>Lactobacillus</i>	<i>plantarum</i>
ATCC19433	<i>Streptococcus</i>	<i>faecalis</i>
Y5SL2	<i>Streptococcus</i>	<i>thermophilus</i>
UV202	<i>Enterococcus</i>	<i>faecalis</i>
OGIRF	<i>Enterococcus</i>	<i>faecalis</i>
CVM99	<i>Enterococcus</i>	<i>faecalis</i>
CVM14	<i>Staphylococcus</i>	<i>aureus</i>
H2Staph	<i>Staphylococcus</i>	unknown
ATCC19114	<i>Listeria</i>	<i>monocytogenes</i>
491	<i>Listeria</i>	<i>innocus</i>

### 3.6 PCR identification of *L. monocytogenes* and API identification of *Salmonella* spp.

#### 3.6.1 PCR identification of *Listeria monocytogenes*

All *Listeria* species were tested using primers specific to *L. monocytogenes*. The primers used were lmo0733-F (5' CGCAAGAAGAAATTGCCATC-3') and lmo0733-R (5'-TCCGCGTTAGAAAAATTCCA -3')<sup>104</sup>. These primers are specific to a *L. monocytogenes* specific gene (*lmo0733*) that has the potential for specific detection of *L. monocytogenes*. Using PCR primers (*lmo0733F* and *lmo0733R*) derived from this gene, a specific fragment of 453 base pairs was amplified from genomic DNA of *L. monocytogenes* strains. Bacterial templates were prepared by heating the bacteria suspended in autoclaved distilled water at 100 °C for 10 minutes. They were then centrifuged at 14000 rpm for 2 minutes. The

supernatant was used for bacterial template. Bacterial template (3  $\mu$ l) was added into 22  $\mu$ l solution containing 200  $\mu$ M of each deoxyribonucleotide triphosphate (dNTP), 2 mM of MgCl<sub>2</sub>, 1 unit of Taq DNA polymerase, 1 pmol of each of the primers and 2.5  $\mu$ l of 10X PCR buffer. The PCR was carried out by a 10-minute denaturation at 94°C, followed by 30 cycles of denaturation (94°C, 1 minute), primer annealing (50° C, one minute), and extension (72°C, 1 minute). After 30 cycles of denaturation, annealing and extension, PCR reactions were then held at 72°C for seven minutes to let Taq polymerase work on the extension of any unfinished ends. The reaction products were kept at 4 °C hereafter. PCR products were visualized under UV light, after staining with ethidium bromide and performing gel electrophoresis on 1.5% agarose gel.

### **3.6.2 Analytical Profile Index (API) identification of *Salmonella* spp.**

The *Salmonella* species that did not give a good reaction with any of the *Salmonella* specific monoclonal antibodies were tested using API to confirm if they were *Salmonella*. For this purpose API identification kit from bioMerieux was used and instructions were followed as recommended.

#### Growing the *Salmonella* strains

The doubtful *Salmonella* strains were grown on plates containing Luria Bertani (LB) and incubated overnight at 37 °C.

#### Preparation of the inoculum

A single well-isolated colony for each strain was removed from the overnight plates and suspended in 5ml of 0.85% NaCl. The colony was carefully emulsified to get a homogenous bacterial suspension.

### Inoculation of the strip

The API strips were inoculated with the bacterial suspension. They were then covered and incubated at 37 °C for 24 hours.

### Reading the strip

The strips were read and the reactions were interpreted according to the table provided with the API identification system. The pattern of reactions obtained is coded into a numerical profile using the specially designed result sheet. Finally a seven-digit profile number is obtained for the 20 tests of each API strip.

### Identification

The seven-digit profile number is used to get the information on the bacteria profile using the identification software provided by bioMerieux.

## **IV RESULTS**

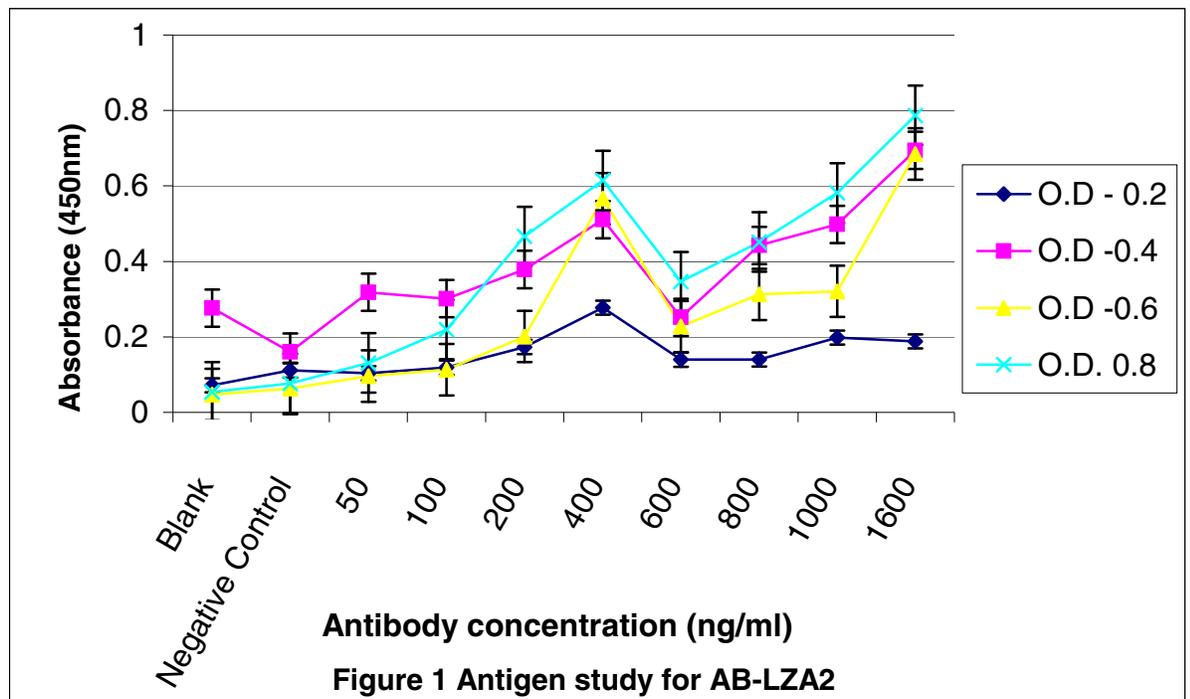
### **4.1 *Listeria monocytogenes***

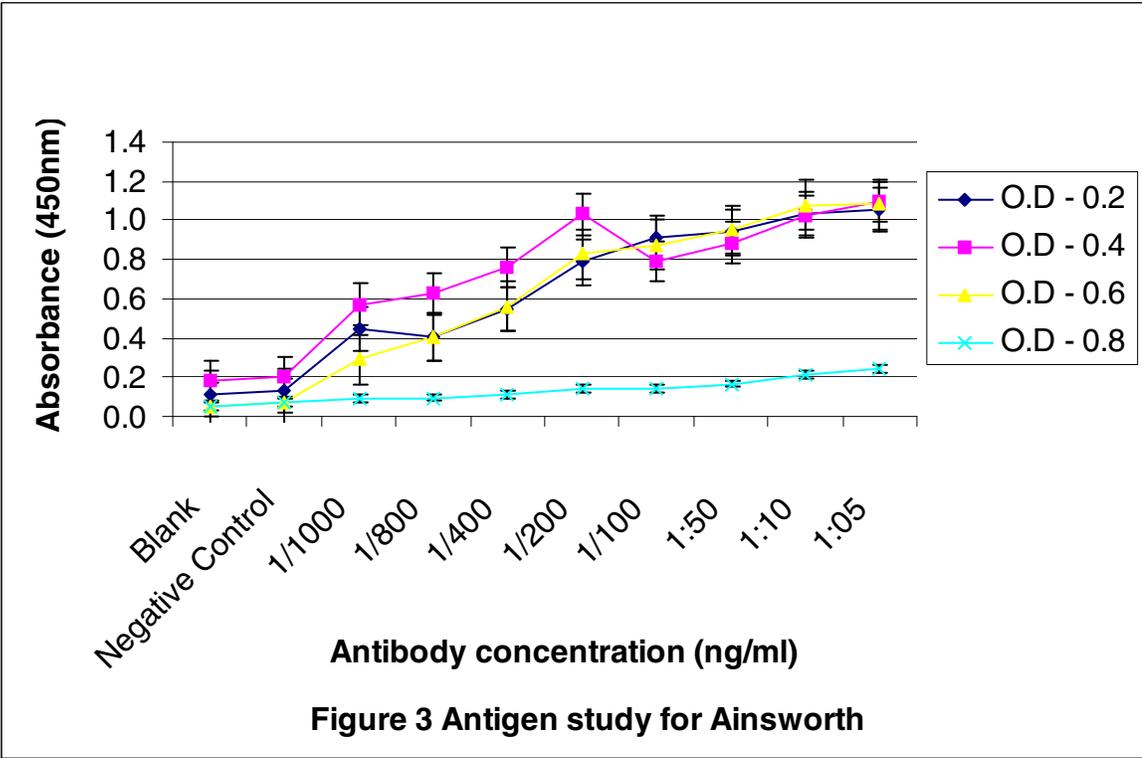
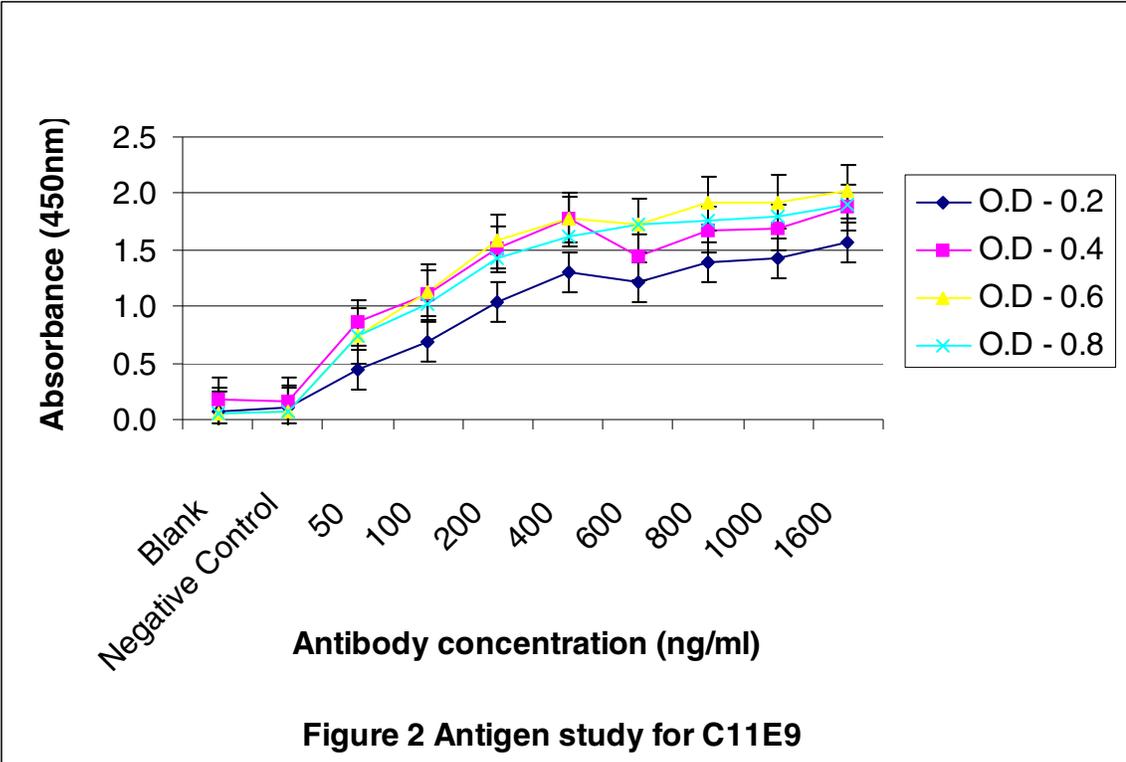
#### **4.1.1 Optimum antibody and antigen concentrations to be used in ELISA**

The eight monoclonal antibodies specific to *L. monocytogenes*, procured from both commercial and academic sources were subjected to a screening process. First of all, study was conducted to select an optimum concentration of the antibody and the antigen to work with.

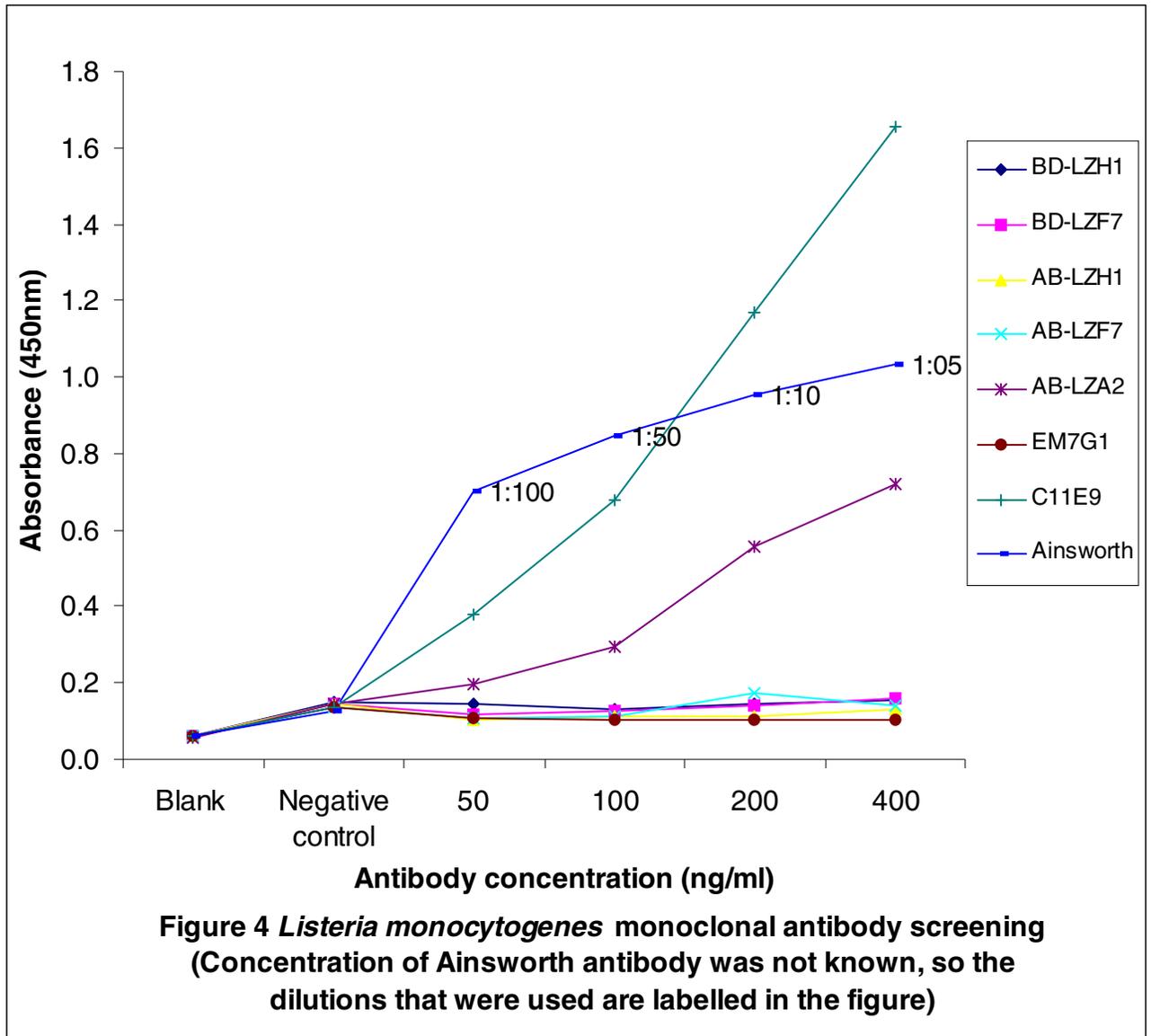
Study was conducted to look for the optimum antigen concentration that should be coated on the plates. For this purpose, the concentrations corresponding to O.D. 0.2, 0.4, 0.6 and 0.8 were tested. Three antibodies, AB-LZA2, C11E9 and Ainsworth were used in this study. Results are shown in

figures 1, 2 and 3. For the antibody AB-LZA2, the antigen concentration corresponding to O.Ds 0.4, 0.6 and 0.8 showed similar trend and similar absorbance values. In case of antibody C11E9, all the four antigen concentrations showed similar trends and absorbance values. For Ainsworth, O.Ds of 0.2, 0.4 and 0.6 showed similar trend but O.D 0.8 gave a very low absorbance. Based on the above data, the antigen concentration corresponding to an O.D of 0.6 was selected to be used in further experiments. Although an O.D of 0.4 also showed similar trend, O.D 0.6 was chosen to ensure good coating of bacteria on the plates. For AB-LZA2 and C11E9, the antibody concentration of 400ng/ml at an antigen concentration of 0.6 gave good absorbance readings. For Ainsworth antibody, the dilution of 1:10 gave good absorbance readings. These antibody concentrations were used in all further experiments.



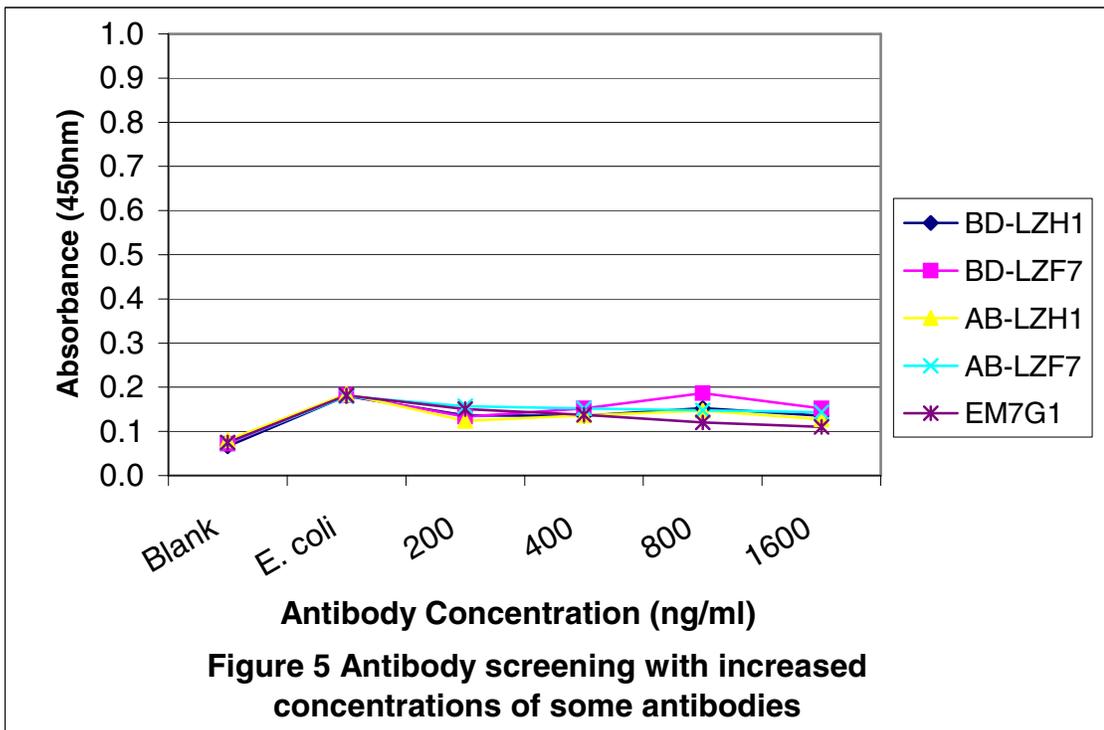


All the eight antibodies were then tested for their optimum concentration and also to see their reactivity. The antibody concentrations of 50ng/ml, 100ng/ml, 200ng/ml and 400ng/ml were tested for all the antibodies except for Ainsworth antibody. Ainsworth antibody was used at dilutions of 1:100, 1:50, 1:10 and 1:5 as its concentration was unknown. The results of this experiment can be seen in Figure 4. Three antibodies out of eight, namely, AB-LZA2, Ainsworth and C11E9 gave good absorbance values when tested by ELISA, but the other antibodies gave an absorbance of less than 0.2 at 450nm.



The experiment was repeated by increasing the concentration for antibodies that gave an absorbance value less than 0.2. The antibody concentrations used were 200ng/ml, 400ng/ml, 800ng/ml and 1600ng/ml. Results are shown in Figure 5. Even increasing the antibody concentration did not improve the absorbance values for the antibodies. It is clear from Figures 4 and 5 that the three antibodies, AB-LZA2, Ainsworth and C11E9 had better

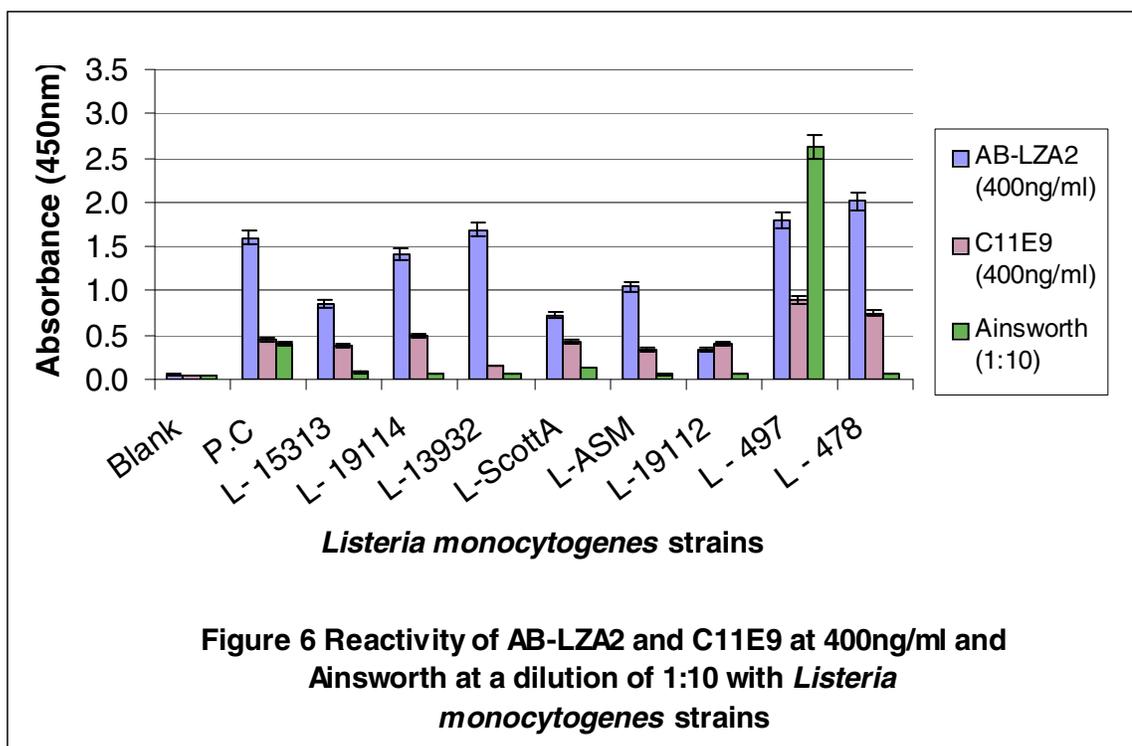
absorbance readings at lower concentrations than the antibodies that had been tested at higher concentrations, thus showing that they reacted with *L. monocytogenes* cells coated on the Microtiter plates, better than the other antibodies. Therefore, the antibodies namely BD-LZH1, BD-LZF7, AB-LZH1, AB-LZF7 as well as EM7G1 were not analyzed any further. All other experiments were performed on AB-LZA2, Ainsworth and C11E9. AB-LZA2 and C11E9 were used in the experiments at 400ng/ml and Ainsworth antibody was used at the dilutions of 1:10.

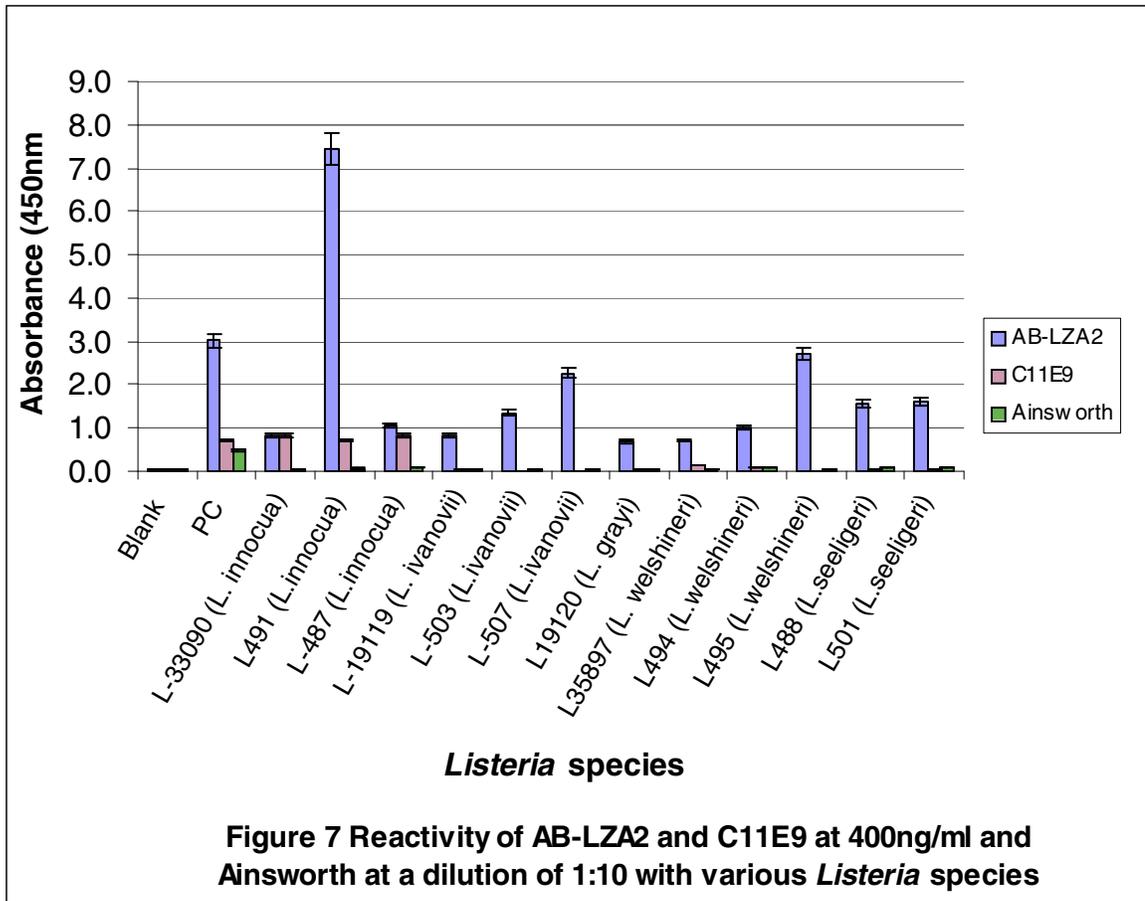


#### 4.1.2 Reactivity of the monoclonal antibodies with *Listeria* strains

The three antibodies namely AB-LZA2, C11E9 and Ainsworth were tested against various *L. monocytogenes* strains. Results are shown in Figure 6. We can see that AB-LZA2 gives highest absorbance values for most of the strains followed by C11E9. Ainsworth antibody did not give noticeable absorbance

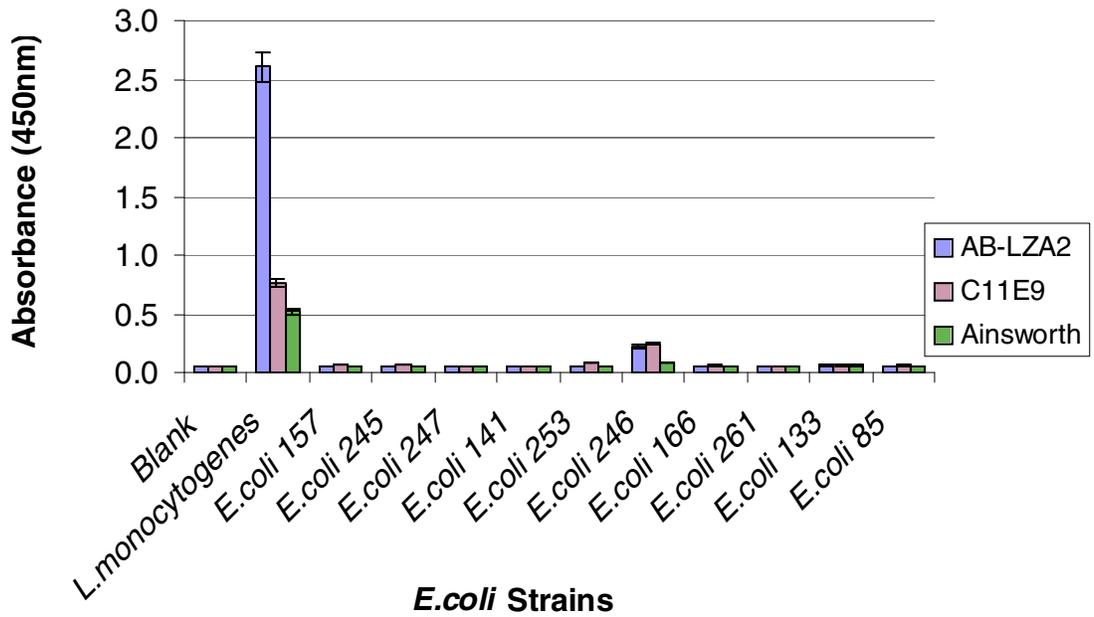
values with *L. monocytogenes* strains except for the strain L-497. Figure 7 shows the reaction of these antibodies with *Listeria* species other than *L. monocytogenes*. From this figure we can see that AB-LZA2 reacted with all the *Listeria* species and not just the *L. monocytogenes*. Ainsworth did not give any considerable absorbance values with these *Listeria* species. C11E9 reacted only with *L. innocua* in addition to *L. monocytogenes* and not with any other *Listeria* species. So Ainsworth did not react well with any of the *Listeria* species including *L. monocytogenes*. C11E9 is more specific to *L. monocytogenes* than AB-LZA2 as it reacts only with one more *Listeria* species other than *L. monocytogenes*.



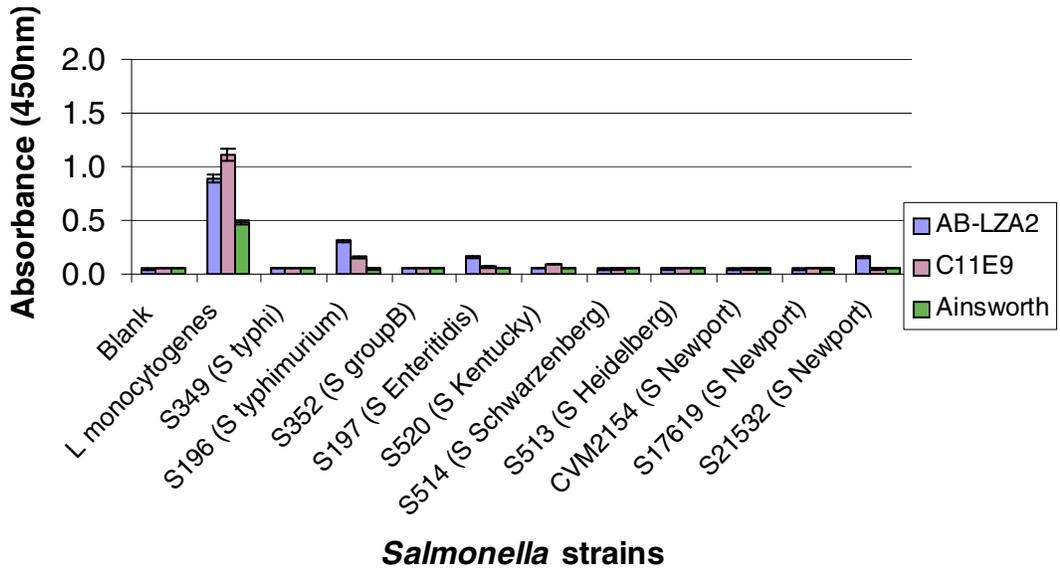


#### 4.1.3 Reactivity of *L. monocytogenes* monoclonal antibodies with other bacterial species.

These antibodies were further tested for their reactivity with other bacterial species which were *Escherichia coli* and *Salmonella* serovars. From the figures 8 and 9 we can see that none of the antibodies showed any noticeable cross reactivity with *E.coli* strains and *Salmonella* serovars tested in this study.

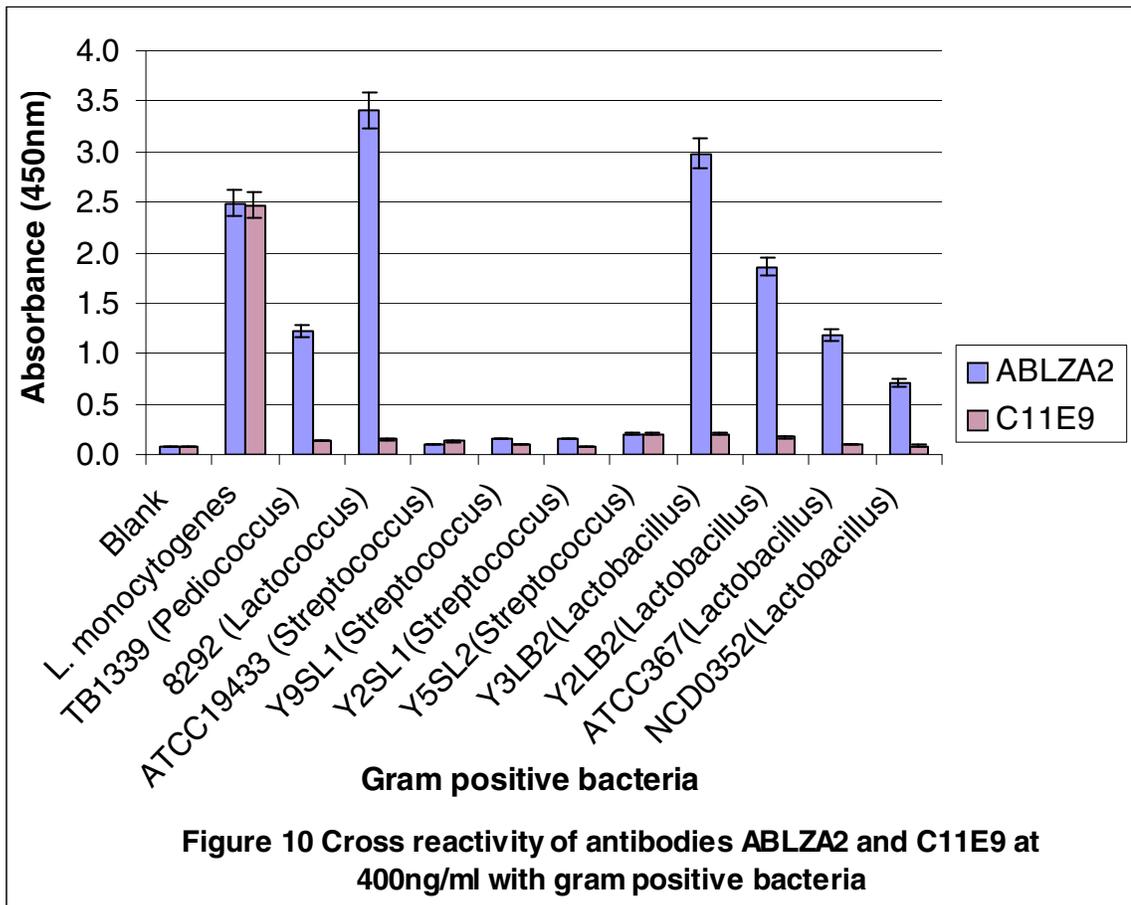


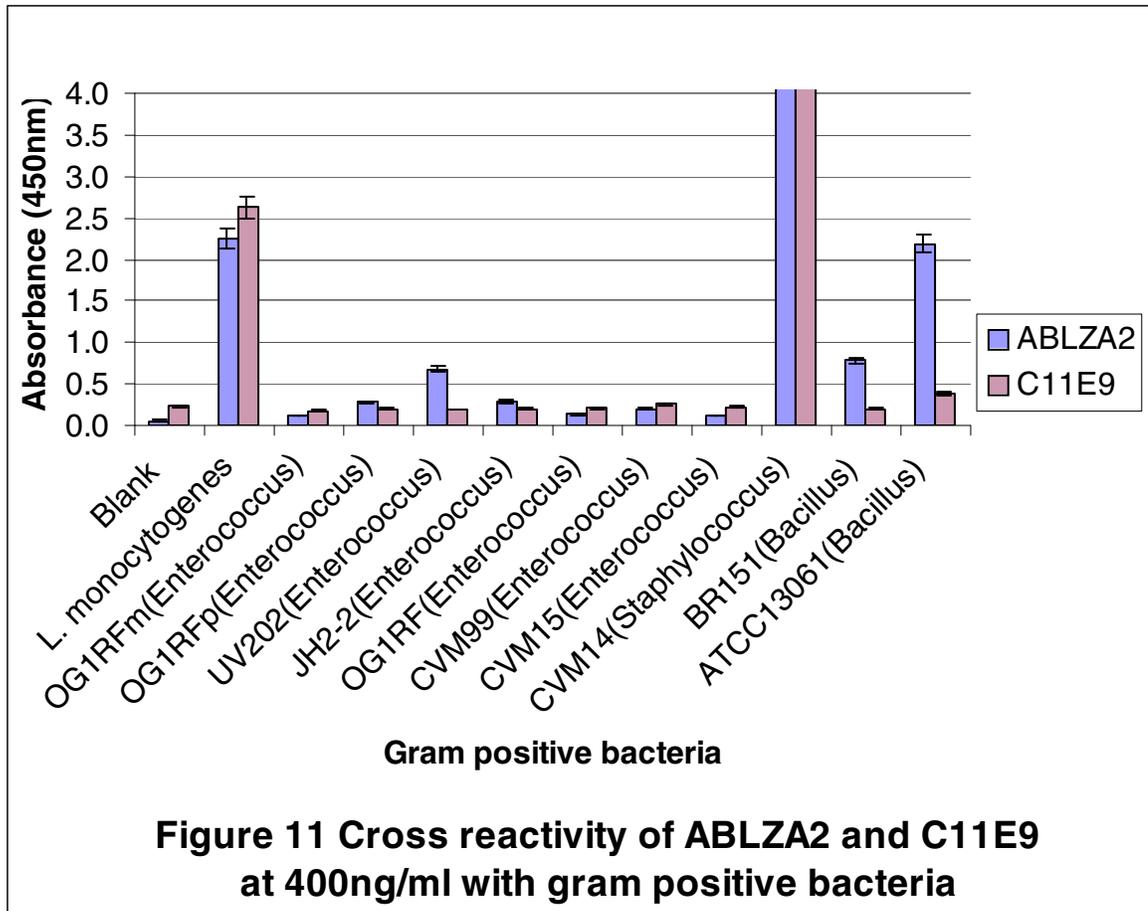
**Figure 8 Reactivity of AB-LZA2 and C11E9 at 400ng/ml and Ainsworth at 1:10 with different *E.coli* strains**



**Figure 9 Reactivity of AB-LZA2 and C11E9 at 400ng/ml and Ainsworth at 1:10 with *Salmonella* strains**

Further cross reactivity studies were conducted on gram-positive bacteria that were Lactic acid bacteria. Ainsworth antibody was not tested any further because of its low reactivity with *L. monocytogenes* and also because of its ability to recognize Listeriolysin(LLO) and not the cell surface proteins. The results summarized in figures 10 and 11 show that ABLZA2 showed considerable cross reactivity with *Lactococcus*, *Lactobacillus*, *Staphylococcus* and one *Bacillus* strain. But C11E9 showed no cross-reactivity with these bacteria except for *Staphylococcus*.

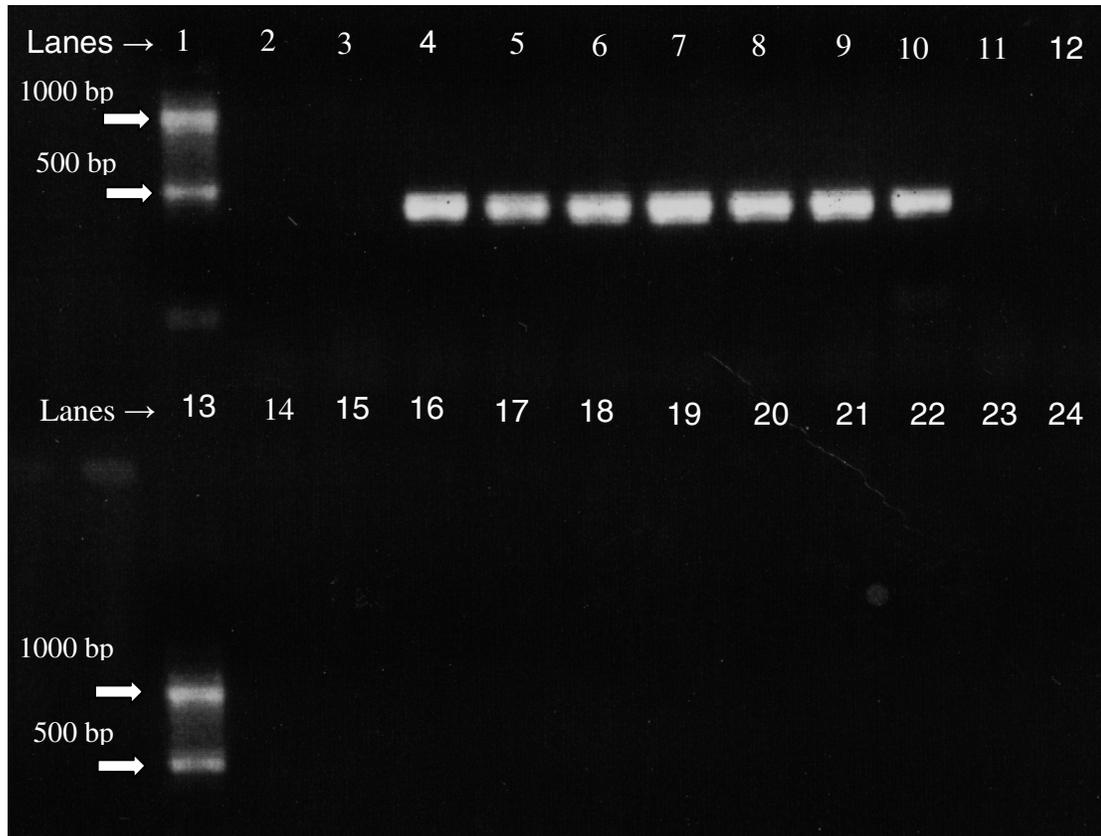




#### 4.1.4 PCR identification of *L. monocytogenes*

In order to be sure that the *L. monocytogenes* strains tested in this study were really *L. monocytogenes*, they were tested by Polymerase Chain Reaction (PCR). All except for one of the strains gave an expected band of 453 base pairs. The *L. monocytogenes* strain L-478 did not give the expected band on the gel thus suggesting that it was not *L. monocytogenes*. All *Listeria* strains other than *L. monocytogenes* were also tested using PCR. This was done to make sure that the other *Listeria* strains used in the study were not *L. monocytogenes*. None of these strains showed any band at the expected position on the gel, thus

suggesting that they were not *L. monocytogenes*. Results are shown in Figure 12.



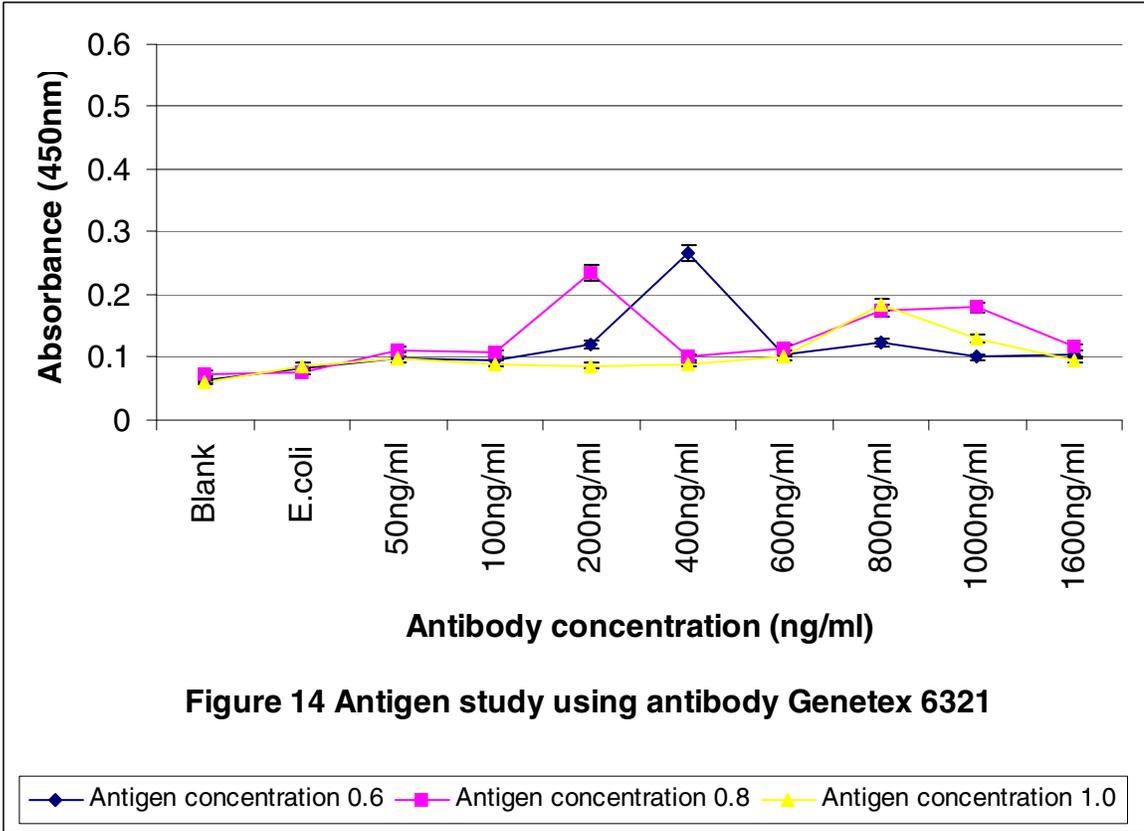
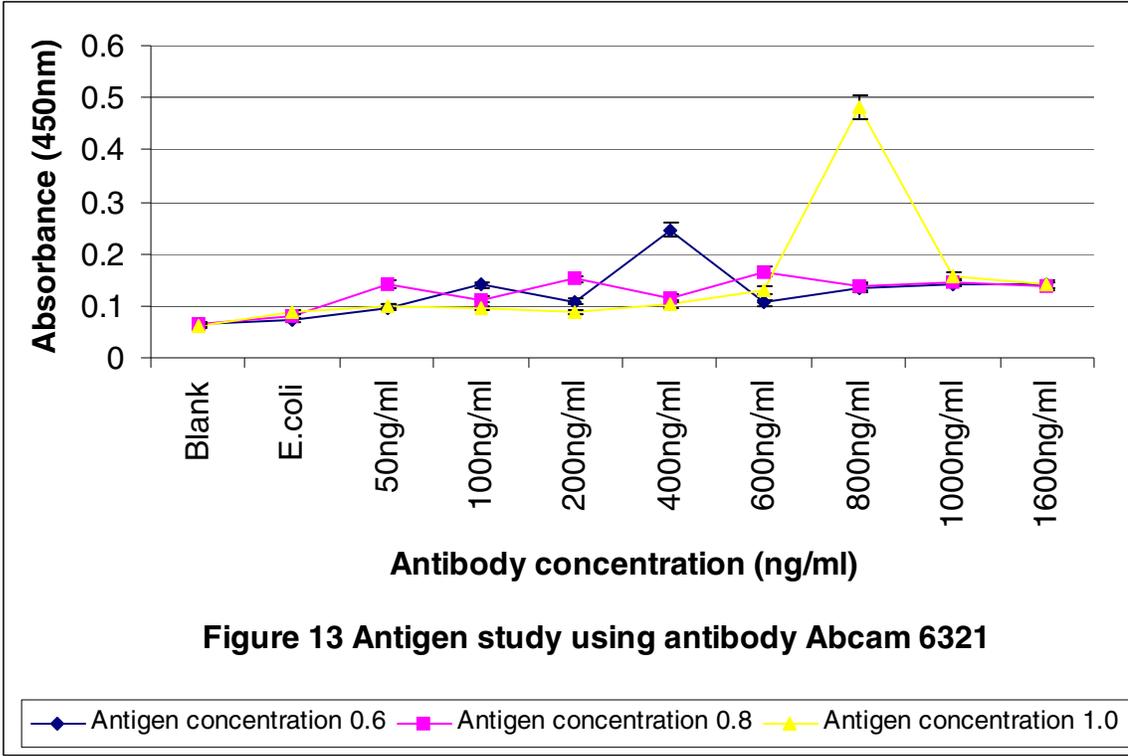
**Figure 12 Agarose Gel picture showing PCR reactions of *Listeria* strains. Lanes 1 and 13 are 100 base pair DNA ladder. Lane 2 is negative control without DNA template. Lane 3 is negative control with *E. coli* DNA. Lanes 4 to 10 are confirmed *L. monocytogenes* strains that gave a band (453 base pairs) near the location of 500 base pair long band of the ladder. Lanes 11, 12 and 14 to 24 contain *Listeria* strains other than *L. monocytogenes*.**

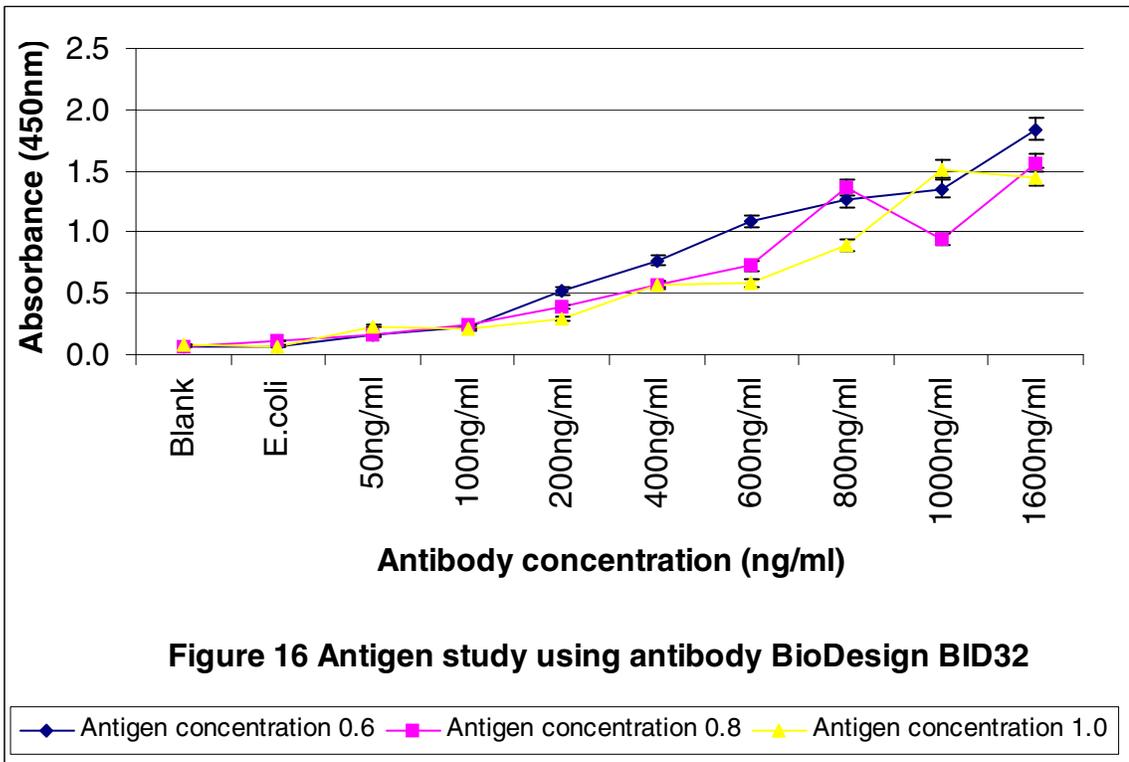
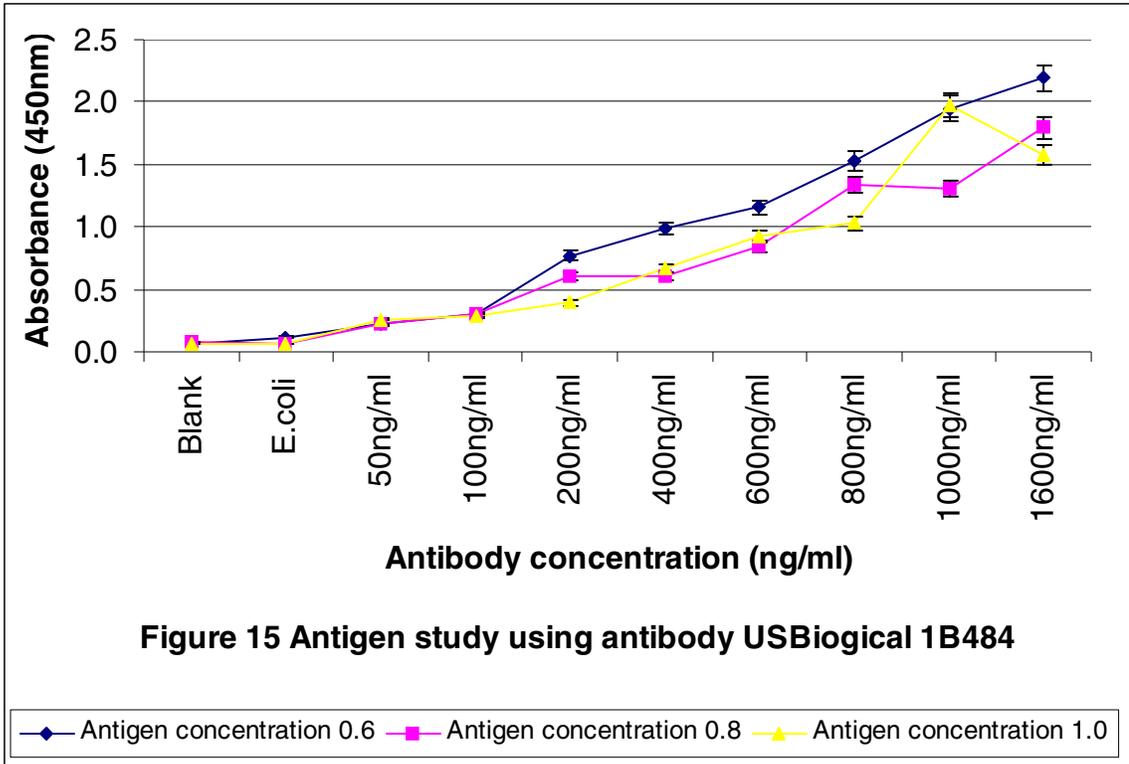
## **4.2 *Salmonella***

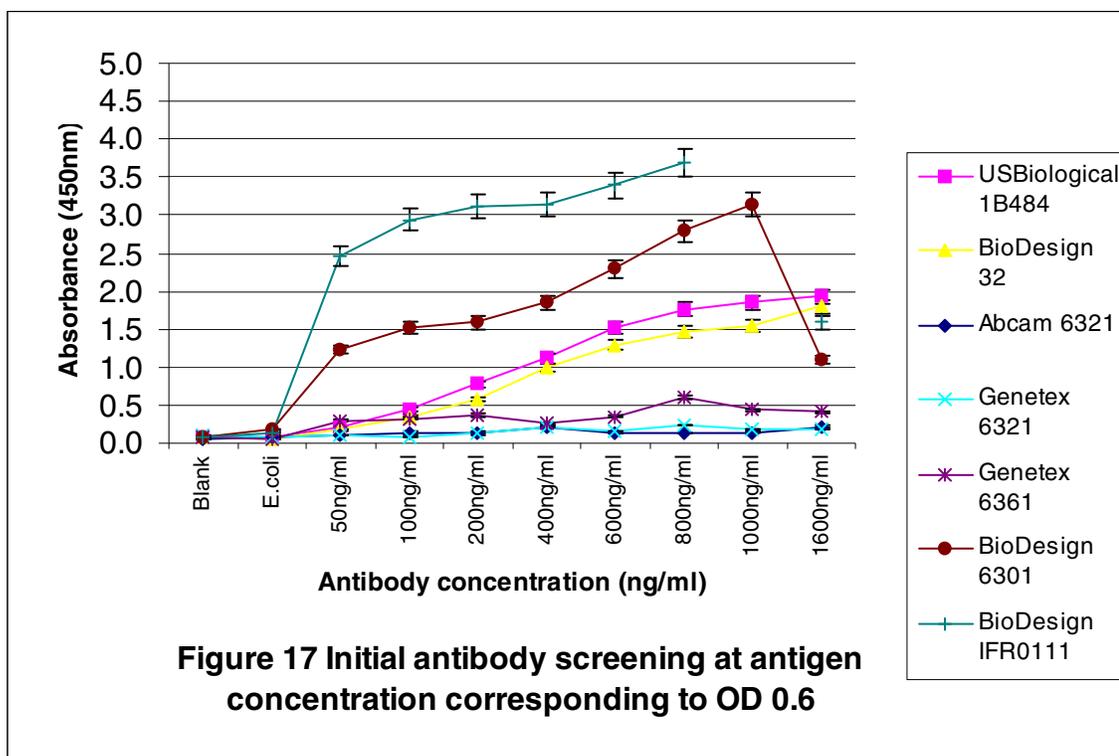
### **4.2.1 Optimum antibody and antigen concentrations to be used in ELISA**

Seven monoclonal antibodies procured from different commercial sources were tested for their optimum concentration to be used in the ELISA assay. Also the optimum concentration of antigen that is the bacteria, *Salmonella* coated on the ELISA plates was tested. For this purpose three antigen concentrations were

tested, these were bacterial concentrations corresponding to O.Ds 0.6, 0.8 and 1.0. Four antibodies namely Abcam 6321, GeneTex 6321, US Biological 1.B.484 and BioDesign BID32 were tested at concentrations ranging from 50ng/ml to 1600ng/ml. The purpose of this study was to look for optimum antigen concentration to be used in the study and also to select the antibodies that gave good reactions. Figures 13, 14, 15 and 16 show the results of antigen study conducted at antigen concentrations corresponding to OD 0.6, 0.8 and 1.0 using four antibodies Abcam 6321, GeneTex 6321, BioDesign BID32 and US Biological 1B484. We can see from the four figures that there was no considerable difference in the response of antibodies by changing the antigen concentration. Therefore the antigen concentration corresponding to OD 0.6 was used in further studies. Results of initial antibody screening done at an antigen concentration corresponding to OD 0.6 are shown in Figure 17. From this study four out of total seven antibodies were chosen to work with. GeneTex 6321, GeneTex 6361 and Abcam 6321 did not give strong absorbance readings at 450 nm in ELISA. So the four other antibodies namely BioDesign BID32, BioDesign 6301, BioDesign IFR0111 and US Biological 1B484 were selected from this experiment.



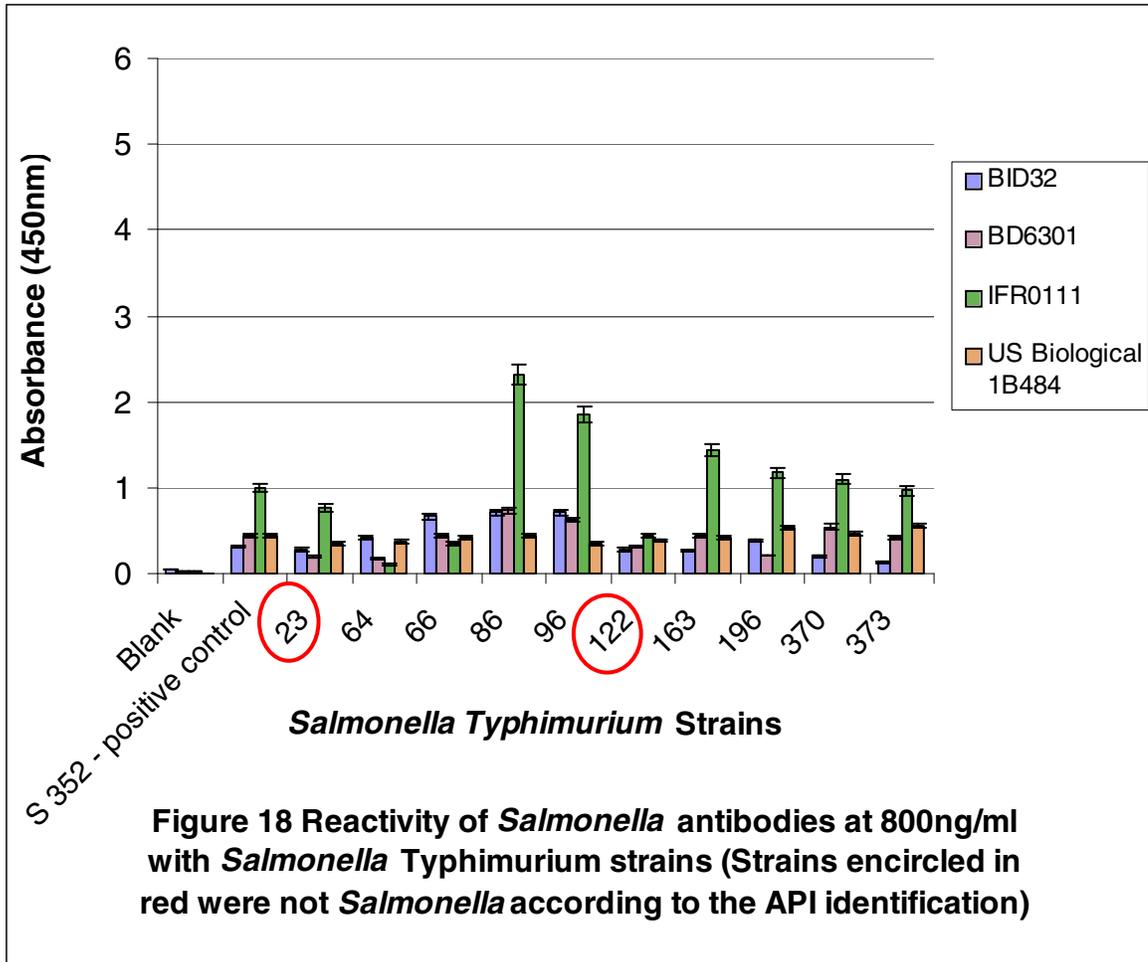


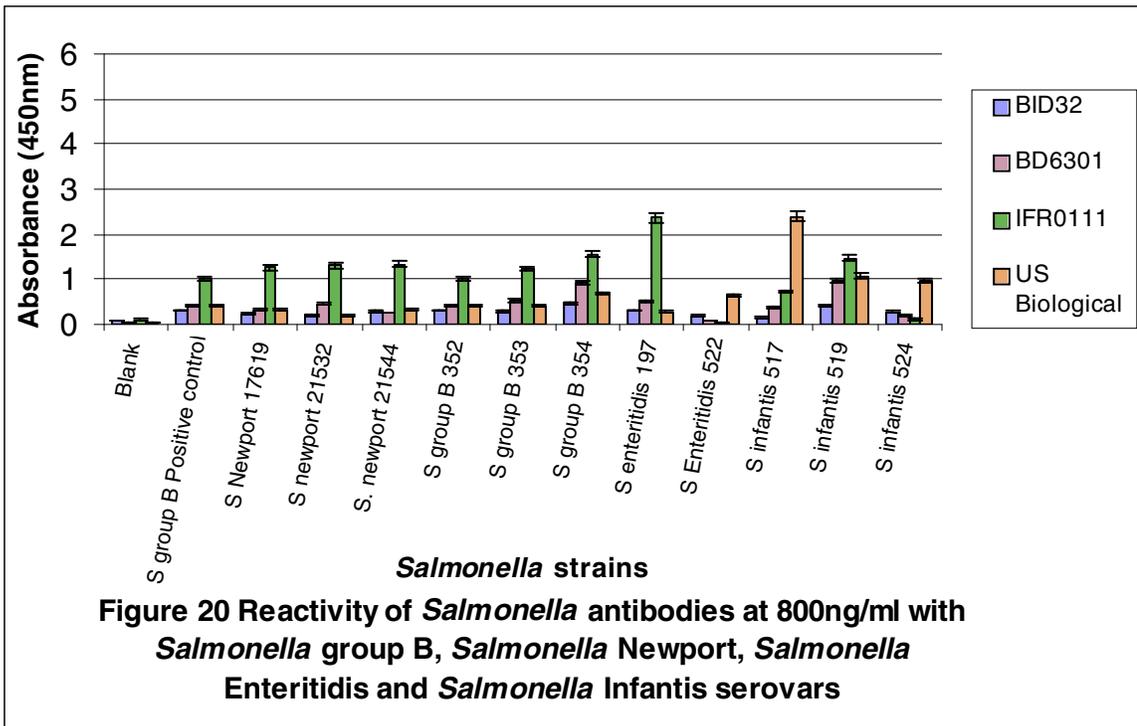
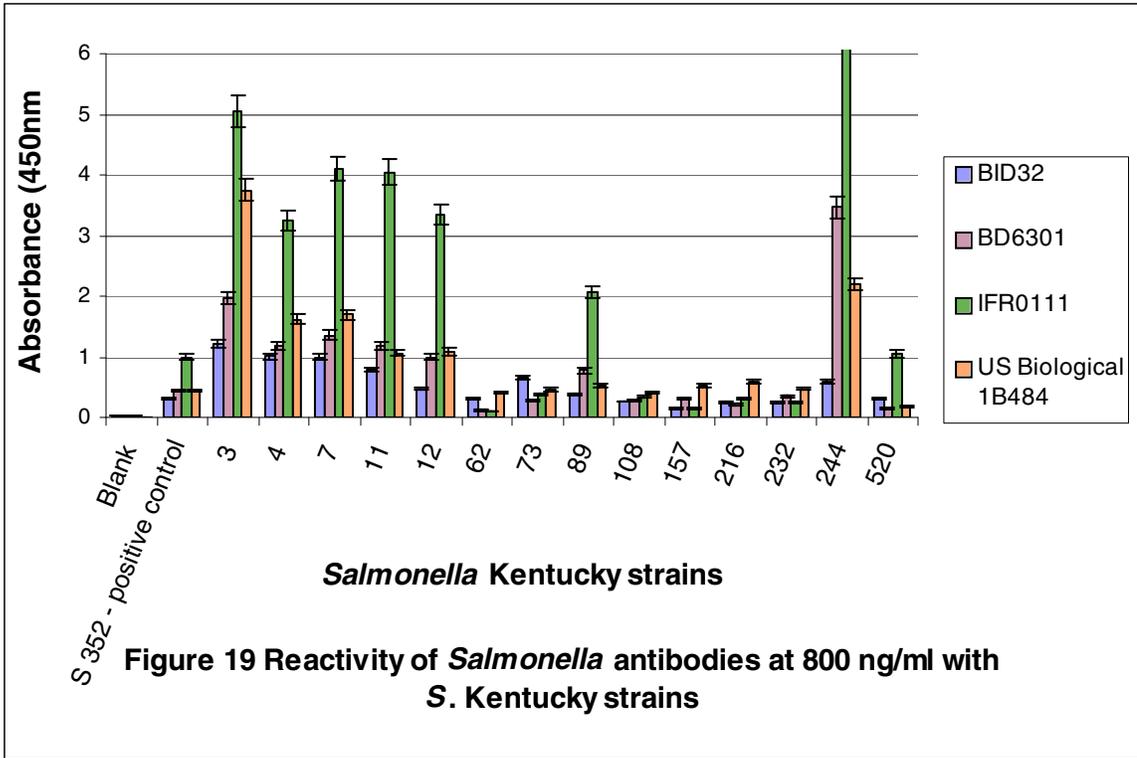


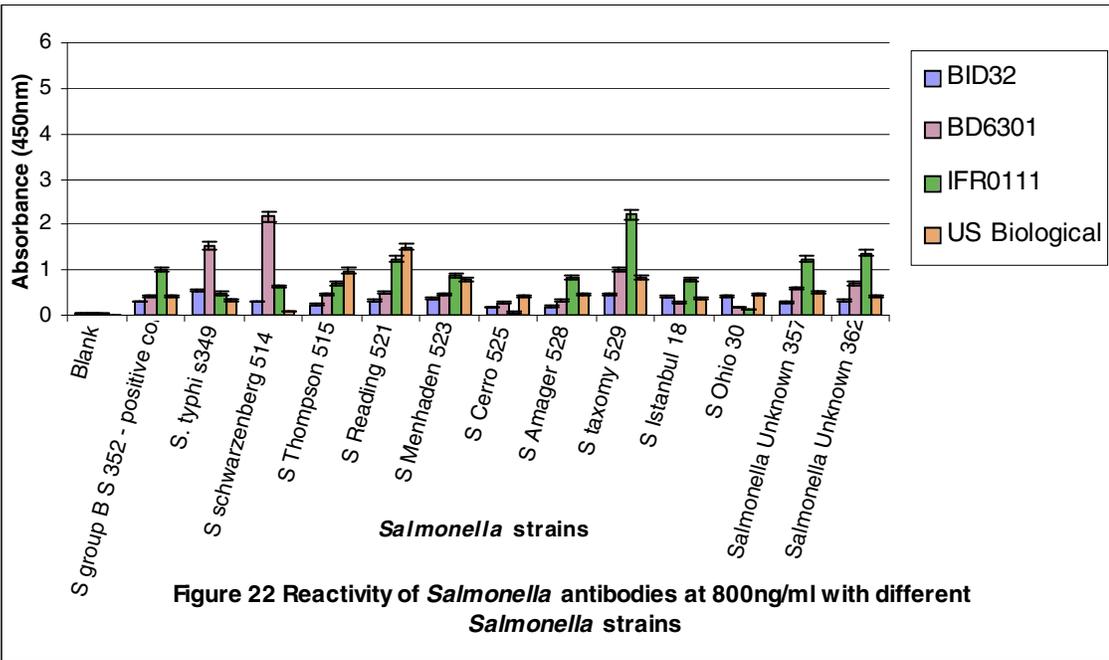
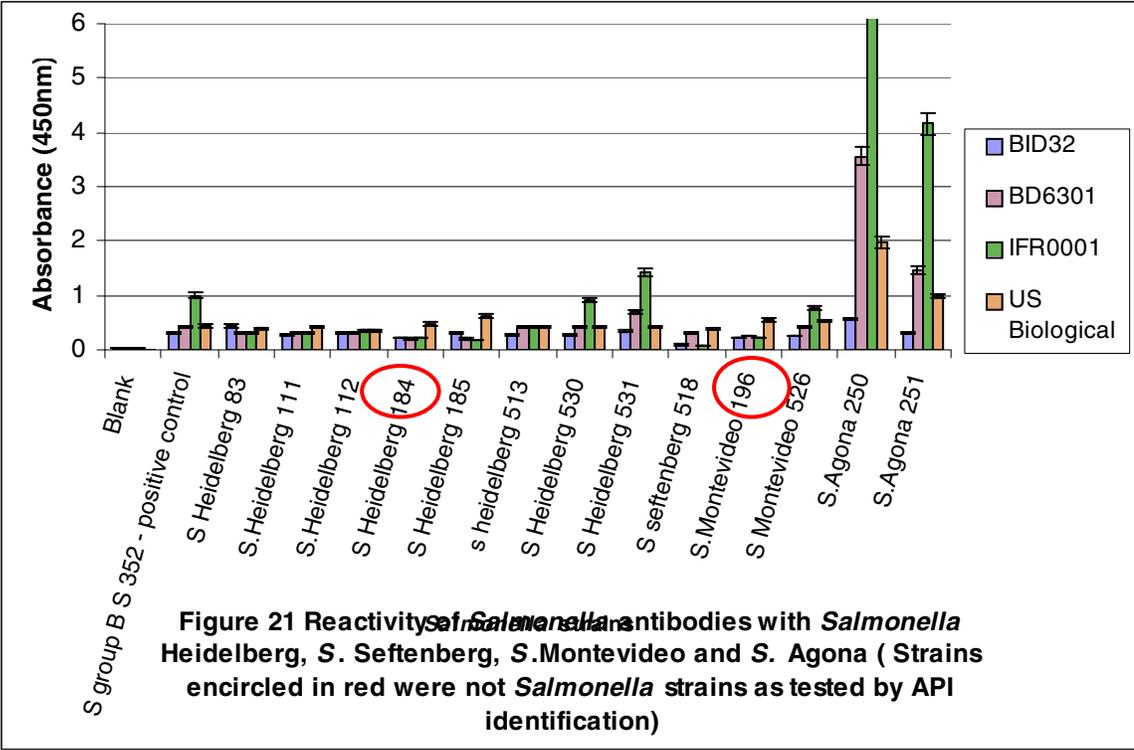
#### 4.2.2 Reactivity of the monoclonal antibodies with *Salmonella* serovars

Next, these four antibodies were tested for their reaction to 60 *Salmonella* strains. There were a total of 20 serovars tested that are listed in Table 4. Results are shown in Figures 18, 19, 20, 21 and 22. BID32 gave a weak signal of less than 1.00 absorbance at 450nm for most of the *Salmonella* strains tested, except for three *Salmonella* Kentucky strains. BD6301 was able to give good absorbance values of one or more than one for six out of total Fourteen *Salmonella* Kentucky strains, one out of three *S. Newport*, all three *Salmonella* group B, one out of two *S. Enteritidis*, one out of three *S. Infantis*, two *S. Agona*, *S. Typhi*, *S. Schwarzengberg* and *S. Taxomy* strains. BioDesign IFR0111 was able to give an absorbance reading of one or more than one for six out of ten *S.*

Typhimurium strains, eight out of Fourteen *S* Kentucky, one out of two *S*. Enteritidis, one out of eight *S*. Heidelberg, two *S*. Agona, one *S*. Reading, *S*. Taxomy and two unknown *Salmonella* strains. US Biological 1B484 gave absorbance values of one or more than one for six out of fourteen *S*. Kentucky, one out of three *S*. Infantis, two *S*. Agona and one *S*. Reading strains.

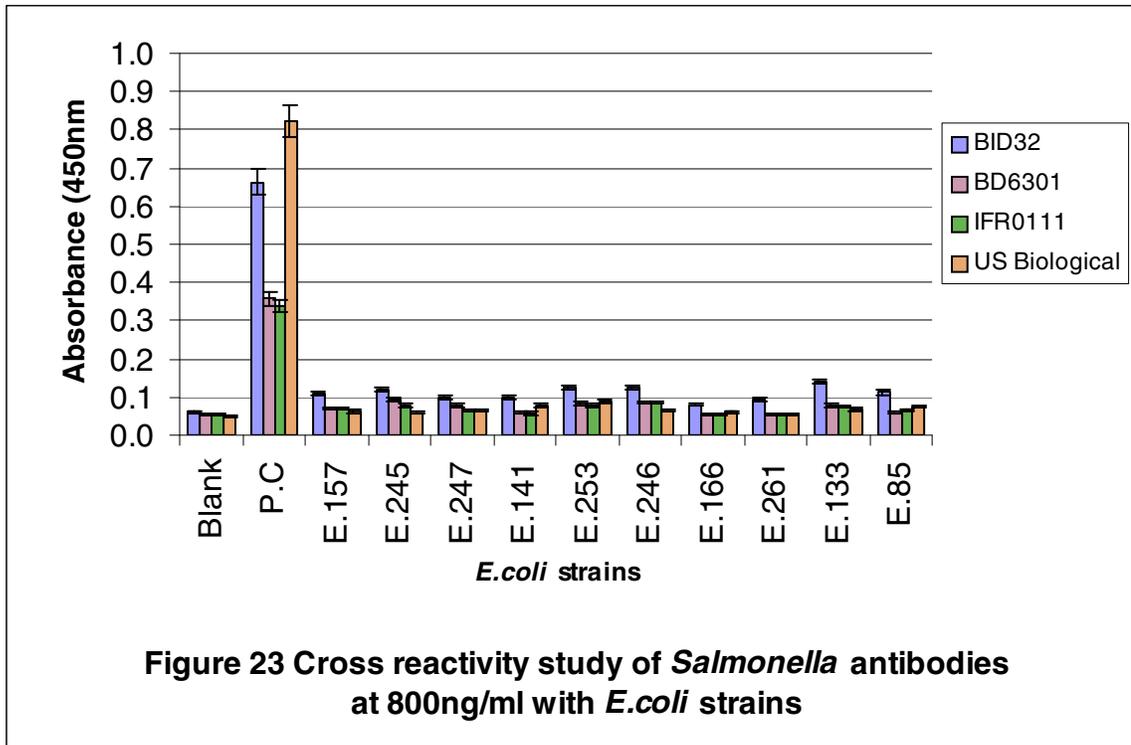


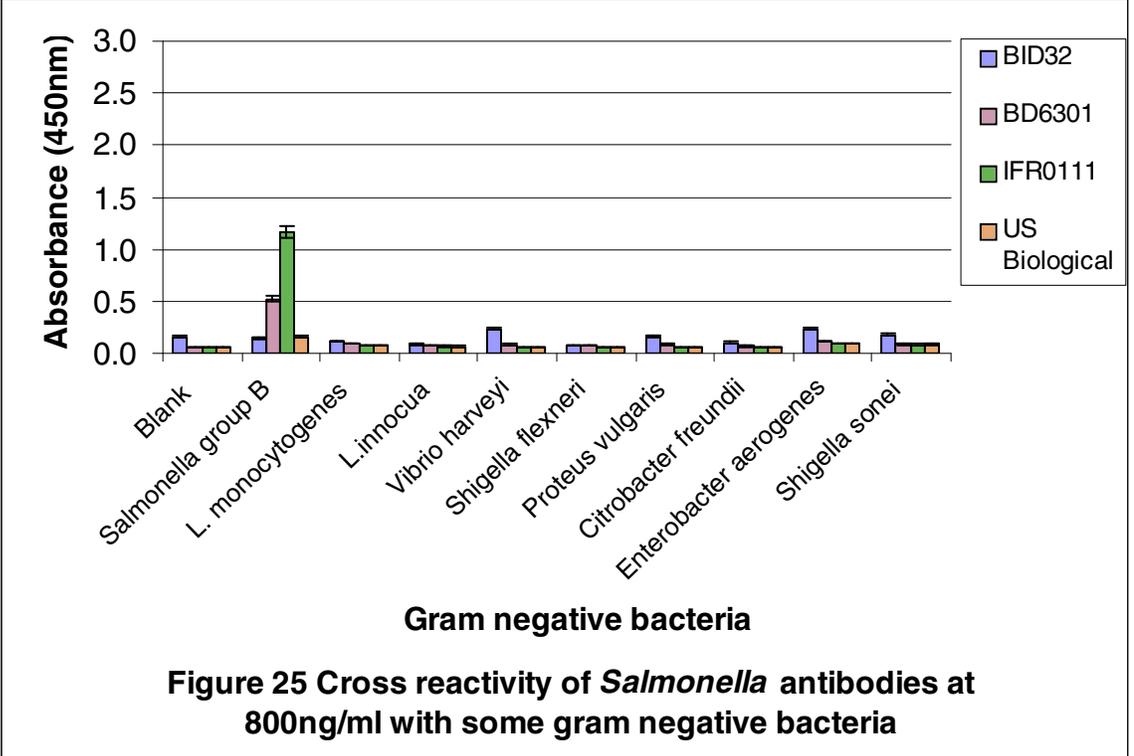
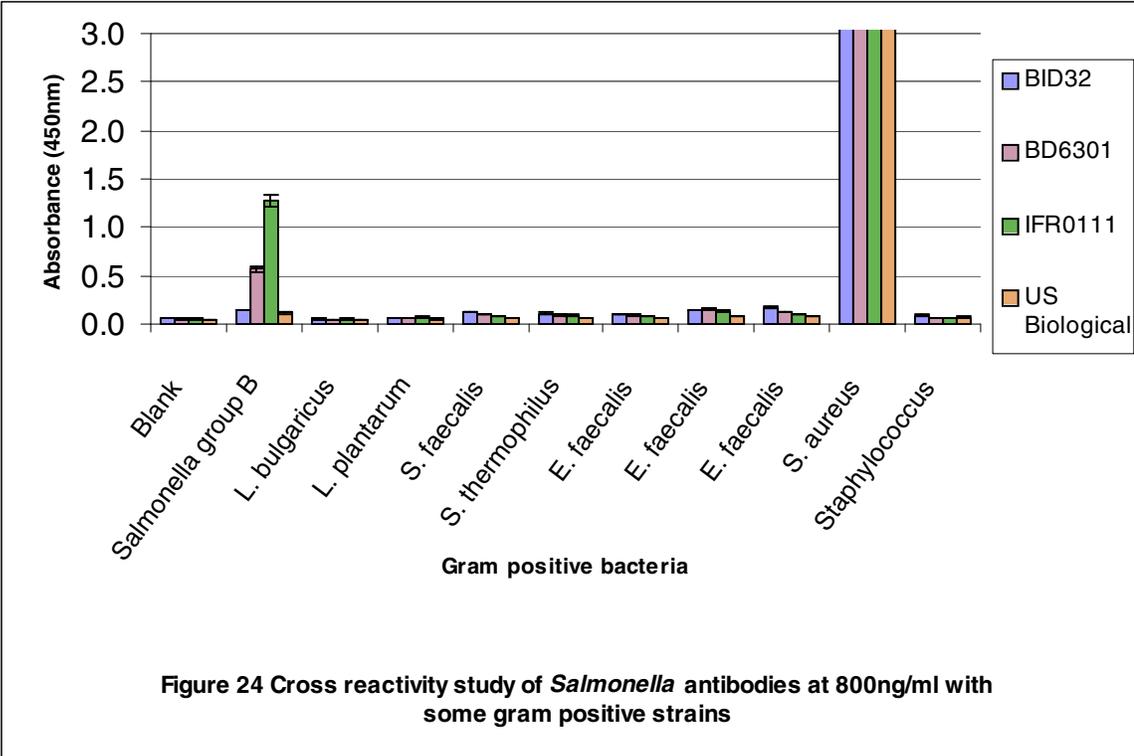




### 4.2.3 Reactivity of *Salmonella* monoclonal antibodies with other bacterial species.

All the four antibodies were also tested for their cross-reactivity with other bacterial species. 10 *E. coli* strains were tested and the results are shown in Figure 23. Also a group of eight other gram-negative bacteria were tested as shown in Figure 25. Nine gram-positive bacteria were also tested and results are shown in Figure 24. Cross-reactivity study showed that these antibodies did not react with any *E.coli* strains considerably and also not with any other Gram-negative bacteria tested. But all the antibodies reacted very strongly with one *Staphylococcus aureus* strain tested in the group of gram-positive bacteria. None of the other gram-positive bacteria gave any considerable absorbance readings with any of the antibodies tested.





#### **4.2.4 API identification of *Salmonella* spp.**

The *Salmonella* strains that did not give good reactions with any of the monoclonal antibodies tested, were subject to an API identification test. Thirty-two out of the total sixty *Salmonella* strains were tested using API strips. The results showed that four out of the thirty-two strains tested were not *Salmonella* strains but were *Hafnia alvei*. These were the ones labeled, *S. Typhimurium* (122 and 23), *S. Montevideo* (196) and *S. Heidelberg* (184).

### **V DISCUSSION**

#### **5.1 *Listeria monocytogenes***

Optimum concentrations of antibodies and antigen, to be used in the ELISA were worked out. From the figures 1, 2 and 3, an antigen concentration corresponding to OD – 0.6 was selected to be coated on the ELISA plates. This concentration was chosen against concentration corresponding to OD – 0.4 to ensure good coating on the ELISA plates because of more number of bacteria using OD – 0.6. The concentration corresponding to OD – 0.8 was ruled out because it gave very low absorbance values for Ainsworth antibody. From figures 1, 2, 3 4 and 5 we can see that the monoclonal antibody concentration of 400ng/ml showed good reactivity. Ainsworth gave a good reading at the dilution of 1:10. So these concentrations were used in the further studies.

Using the above information, initial screening of the antibodies was conducted. Figure 4 shows the response of all the eight antibodies at concentrations of 50, 100, 200 and 400 ng/ml. Only the antibodies AB-LZA2, C11E9 and Ainsworth gave considerable absorbance readings. Another

experiment conducted with increased concentrations of the antibodies that did not give good signals in the previous experiment, also did not give better results. In figure 5 we can see that even with increased antibody concentrations, the five antibodies did not give any good absorbance readings. Thus, the five antibodies; BD-LZH1, BD-LZF7, AB-LZH1, AB-LZF7 and EM7G1 were rejected and not used in further studies.

These antibodies were then tested against eight *L. monocytogenes* strains. . Based on the overall reaction pattern, the reactions were divided into three categories, High (1.0 or higher), Intermediate (0.40 to 0.99) and Low (Lower than 0.40). From Figure 6 we can see that the antibody, AB-LZA2 gave absorbance readings of more than 0.4 for almost all the *L. monocytogenes* strains except for L-19112. C11E9 gave absorbance readings close to 0.4 for six strains and crossed the value of 0.4 for only two strains; L-497 and L- 478. It gave the lowest reading with L- 13932. Ainsworth gave very inconsiderable readings for all the strains except for L- 497. Results are summarized in table 12

Table 12 Reactions of *L. monocytogenes* specific monoclonal antibodies to *L. monocytogenes* strains

Antibody	Number of <i>L. monocytogenes</i> antibodies in each category		
	Low	Intermediate	High
AB-LZA2	1	0	7
C11E9	0	6	2
Ainsworth	7	0	1

So we can say that AB-LZA2 and C11E9 recognized *L. monocytogenes*, with AB-LZA2 showing better reactivity. Ainsworth antibody was unable to recognize seven out of eight *L. monocytogenes* strains tested thus creating a serious doubt about its ability to recognize *L. monocytogenes*. One reason could be that unlike the other two antibodies; AB-LZA2 and C11E9 that recognize the cell surface proteins, Ainsworth antibody recognizes the antigen Listeriolysin (LLO). Since whole cells were coated on ELISA plates, Ainsworth antibody was unable to recognize them. It gave a good reaction with just one *L. monocytogenes* strain, i.e L-497 and the reason could be that this strain produces LLO, that Ainsworth antibody was able to recognize.

These three antibodies were also tested against some *Listeria* species other than *L. monocytogenes*, to test their specificity to recognize just the *L. monocytogenes*. Figure 7 shows that AB-LZA2 reacted well with all the species tested, thus telling us that it was not specific for recognizing *L. monocytogenes*. C11E9 recognized only *L. innocua* strains from the strains tested. The producer of C11E9, Dr Arun Bhunia had reported the reactivity of this antibody to two *Listeria* species; *L. monocytogenes* and *L. innocua*. He reported two surface proteins of different sizes on these two *Listeria* species, to which the antibody C11E9 binds<sup>105</sup>. Ainsworth antibody did not show any considerable reaction with any of the strains tested.

Results of the PCR done to confirm that *L. monocytogenes* strains used in the study were really *L. monocytogenes*; showed that L-478 was not *L. monocytogenes* as it did not give a band at the desired position on the gel. It

could be *L. innocua* because C11E9 reported to recognize only *L. monocytogenes* and *L. innocua* gave a considerable reaction with this strain.

In the food industry, we are concerned about testing for *L. monocytogenes* that is a major cause of food borne illness. AB-LZA2 could identify the presence of *Listeria* species in general but not *L. monocytogenes* specifically. C11E9 reacted with *L. monocytogenes* and *L. innocua*, thus proving that it was more specific than AB-LZA2, and is a better choice to be used in a biosensor made for food industry.

These antibodies were also tested for their cross-reactivity to *E. coli* and *Salmonella* strains. None of the antibodies showed any reactions with any of these gram-negative strains (Figures 8 and 9). But when AB-LZA2 and C11E9 were tested with some gram-positive bacteria, AB-LZA2 was found to react with *Pediococcus*, *Lactococcus*, *Lactobacillus*, *Staphylococcus* and *Bacillus* strains. Other than giving a near 0.5 reading for one *Bacillus* strain and reacting strongly with *Staphylococcus*, C11E9 did not react with any other gram-positive bacteria.

So out of the three antibodies tested for *L. monocytogenes*, Ainsworth did not recognize *L. monocytogenes* whole cells. It was reported to recognize LLO, and thus could not recognize the whole cells coated on the ELISA plates. For the production of this biosensor, it was needed that the antibody could recognize the whole cell, so this antibody could not be used in the biosensor. AB-LZA2 was more sensitive i.e. gave better absorbance readings for *L. monocytogenes* as compared to C11E9, but it was not specific to recognizing *L. monocytogenes*. It cannot be used in a biosensor designed to sense the presence of *L.*

*monocytogenes* in food, because its probability of falsely detecting *L. monocytogenes* is much higher. This is because it can react to almost all *Listeria* species and also some gram-positive bacteria fairly strongly. C11E9 recognized *L. monocytogenes* and *L. innocua* but not any other *Listeria* strain. Its cross-reactivity with other bacterial species was also low, as it reacted with only one gram-positive strain and none of the gram-negative strains.

## **5.2 Salmonella**

Seven monoclonal antibodies specific to *Salmonella* species were procured from commercial sources and tested for their ability to recognize *Salmonella* species without cross-reacting with other bacteria. Four antibodies, Abcam 6321, Genetex 6321, US Biological 1B484 and BioDesign BID32 were tested at concentrations ranging from 50ng/ml to 1600ng/ml for antigen concentrations corresponding to ODs 0.6, 0.8 and 1.0. Figures 13, 14, 15 and 16 show that the antigen concentrations showed almost same reactions for all the four antibodies. So the antigen concentration corresponding to OD 0.6 was chosen to be used in the studies. Antibody concentration of 800 ng/ml was used in further experiments.

Initial antibody screening conducted for the seven antibodies, rejected three antibodies because they gave inconsiderable absorbance readings as compared to the other four antibodies tested. The three rejected antibodies were Abcam 6321, Genetex 6321 and Genetex 6361. The other four antibodies, US Biological 1B484, BioDesign 6301, BioDesign BID32 and BioDesign IFR0111

gave absorbance readings of more than 1.0 at 800ng/ml, and were selected to be used in further screening.

These antibodies were then tested for their reactivity to 60 *Salmonella* strains that covered 20 *Salmonella* serovars. The absorbance readings ranged from a very low of 0.06 to high 5.05. Some antibodies gave very strong reactions that could not be read, so the highest reading taken here was the one that could be read by the ELISA reader. Based on the overall reaction pattern, the reactions were divided into three categories, High (1.0 or higher), Intermediate (0.60 to 0.99) and Low (Lower than 0.60). Results are summarized in Table 13. By API identification, it was found that four out of the sixty strains tested were not *Salmonella* and they have been marked with a red circle on the figures. Based on the above mentioned categories, the four antibodies showed the following reaction pattern with forty-six *Salmonella* strains tested.

Table 13 Reactions of *Salmonella* antibodies to different *Salmonella* strains

Antibody	Number of <i>Salmonella</i> strains in each reaction category		
	LOW	INTERMEDIATE	HIGH
BID32	48	6	2
BD6301	38	8	10
IFR0111	19	10	27
US Biological	37	9	10

So we can see from the above table that IFR0111 had high reaction with maximum number of strains. BID32 was the least reactive, giving a low reaction with forty-eight out of fifty-six strains, followed by a little BD6301 that gave a low reaction with thirty-eight out of fifty-six strains tested. US Biological was almost the same as BD6301. So from the above results, IFR0111 seemed like the antibody recognizing maximum number of *Salmonella* strains. IFR0111 gave a low reaction with two out of eight *S. Typhimurium* strains, six out of fourteen *S. Kentucky* strains, one out of two *S. enteritidis*, one out of three *S. Infantis*, five out of seven *S. Heidelberg*, one *S. Seftenberg*, *S. Typhi*, *S. Cerro* and *S. Ohio* strains. So out of twenty serovars of *Salmonella* tested, IFR0111 was able to recognize almost sixteen serovars. This shows that this antibody has a broad-spectrum reactivity with *Salmonella* and can be a good choice for the biosensor. The aim of the biosensor is to recognize not a particular *Salmonella* serovar but any *Salmonella* that might be present in the sample.

When all these antibodies were tested against ten *E.coli* strains to check their cross-reactivity, none of the antibodies showed considerable absorbance readings. Comparatively, BID32 gave an absorbance value of more than 0.1 as opposed to other antibodies that gave readings of less than 0.1. But still the absorbance values given by BID32 were not considerable enough as compared to the reactions with positive control. So, we can say that none of the antibodies cross-reacted with any *E.coli* strain.

Cross-reactivity was also tested against some other Gram-negative bacteria. Results shown in Figure 25 show that none of the antibodies showed

any cross reactivity with any of the strains tested. Figure 24 shows the results of cross-reactivity study conducted against some Gram-positive bacteria. All except one gram-positive bacteria strain showed no considerable reactivity to any of the antibodies. *Staphylococcus aureus* reacted very strongly with all the antibodies.

## **VI CONCLUSION**

Food borne pathogens are a serious threat to human health and measures need to be taken to control their spread. Many outbreaks of food borne illness occur due to consumption of food prepared in a single facility and distributed amongst a large number of consumers. To control any future outbreaks, it is very important that any contamination in the food is detected in a timely manner. Rapid detection methods for food borne pathogens can play a very important role in improving the food safety. The monoclonal antibodies specific to *Listeria monocytogenes* and *Salmonella* spp were tested in this study to be used in biosensors for rapid detection of these bacteria. For *L. monocytogenes*, the monoclonal antibody C11E9 was found to be most suitable. This antibody was able to recognize *L. monocytogenes* and *L. innocua* without cross-reacting with other *Listeria* spp and other bacterial species tested. In case of monoclonal antibodies tested for *Salmonella*, the antibody IFR0111 was able to recognize the maximum number of *Salmonella* strains. It was also able to recognize sixteen out of twenty serotypes tested. The cross-reactivity of this antibody with other bacterial strains was almost negligible. Future studies may include testing some more antibodies specific to *Salmonella*. IFR0111 was the best antibody from the antibodies tested in this study, but it was able to react

strongly with only 48% of the strains tested. Also more studies need to be conducted to see the sensitivity of these antibodies towards recognizing the pathogens. It is especially important in case of *L. monocytogenes*, for which zero-tolerance is practiced in many countries.

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