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Microplate Immunocapture Coupled with LAMP-BART and Selective Plating for the Rapid Detection of *Salmonella* Infantis in Dry Dog Food and Treats

A Thesis by

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Chapman University Orange, CA

Schmid College of Science and Technology

Submitted in partial fulfillment of the requirements for the degree of

Master of Science in Food Science

May 2021

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December 2018

Microplate Immunocapture Coupled with LAMP-BART and Selective Plating for the Rapid Detection of *Salmonella* Infantis in Dry Dog Food and Treats

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by Danielle Kristine Rosen

DEDICATION

This thesis is dedicated to my parents that always offered words of encouragement throughout the long research process. To my supportive sister and nephews that always cheered me on. To my friends that allowed me to talk about my research incessantly and got to know the process as well as I did. And to all of the classmates and colleagues that always offered great advice, support, and a helping hand.

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ABSTRACT

Microplate Immunocapture Coupled with LAMP-BART and Selective Plating for the Rapid Detection of *Salmonella* Infantis in Dry Dog Food and Treats

by Danielle Kristine Rosen

The objective of this study was to use microplate immunocapture (IC) to reduce the enrichment time required for detection of *Salmonella* in pet food with loop-mediated isothermal amplification with bioluminescent assay in real time (LAMP-BART) and selective plating on XLD. Dog food and pig ear treats were inoculated with *Salmonella* Infantis at concentrations of 10^0-10^4 CFU/25 g, followed by a 3-h enrichment, then microplate IC and LAMP-BART or microplate IC and selective plating on XLD. Another set of samples underwent a traditional 24-h enrichment followed by LAMP-BART or selective plating. Based on the results of three independent trials, microplate IC followed by selective plating enabled detection of *Salmonella* in 100% of dog food and treat samples tested, including at levels as low as 10^0 CFU/25 g. Microplate IC coupled with LAMP-BART enabled detection of *Salmonella* in dog food and treat samples down to levels of 10^0 CFU/25 g, with overall detection rates of 90-93%. These results indicate high potential for microplate IC to be used in place of the traditional 24-h enrichment step, enabling detection of *Salmonella* in complex matrices when coupled with LAMP-BART or selective plating.

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LIST OF ABBREVIATIONS

AAFCO-Association of American Feed Control Officials

BAM-Bacteriological Analytical Manual

BART-Bioluminescent Assay in Real -Time

CDC-Center for Disease Control

FDA-Food and Drug Administration

FD&C-Federal Food, Drug, and Cosmetic Act

GMP-Good Manufacturing Practices

GRAS-Generally Ruled as Safe

HACCP-Hazard Analysis and Critical Control Points

IC-Immunocapture

iNAAT-Isothermal Nucleic Acid Amplification Technologies

LAMP-Loop-Mediated Isothermal Amplification

LOD-Limit of Detection

PCR-Polymerase Chain Reaction

PP_i-Inorganic Pyrophosphate

SOI-Standard of Identity

SOP-Standard Operating Procedures

USDA-United States Department of Agriculture

VIDAS-ICS-Vitek immune diagnostic assay system

1. Introduction

Salmonella enterica is the leading bacterial cause of foodborne illness in the United States, responsible for approximately 1.2 million illnesses, 23 thousand hospitalizations and over 450 deaths each year (CDC 2019). Animal products are major sources of *Salmonella* and common foods associated with salmonellosis are eggs, poultry, meat, unpasteurized milk or juice, cheese, fruits, and vegetables (FDA 2018a). Animal products like poultry and beef and grain products make up approximately 67% of foods associated with *Salmonella* contamination, compared to 13% of produce and nuts, and 20% "other foods," which would include pet foods (CDC 2013). Animal products also make up a bulk of the ingredient list of many pet food products. The infective dose of Salmonella is fewer than 1,000 cells, and after ingesting or handling contaminated food products, symptoms of salmonellosis occur within 6-48 hours and last approximately 4-7 days. Symptoms are generally diarrhea, vomiting, abdominal cramping, and fever. In severe cases, specifically with immunocompromised individuals, an untreated *Salmonella* infection can lead to death due to dehydration and electrolyte imbalance (FDA 2012).

Salmonella is a highly resistant bacterium that can survive in low moisture foods and desiccation conditions for long periods (Lambertini et al. 2016). For example, Lambertini et al. (2016) found that 8 *S. enterica* serotypes (*S.* Anatum, *S.* Typhimurium, *S.* Infantis, *S.* Mbandaka, *S.* Derby, *S.* Newport, *S.* Ohio, *S.* Montevideo, *S.* Bredeny) can survive in dry dog food products for up to 7 months past the "best by" or expiration date of the food. Some ingredients in dry pet food products, like lipid coatings and flavorings, are applied after thermal processing, which increases the risk of pathogen contamination (Lambertini et al. 2016). The main ingredients in dry dog food and treats are also often common sources of *Salmonella*, making the products vulnerable to contamination from individual ingredients during processing. When a

contaminated product is ingested, *Salmonella* infection can lead to illness and/or death in pets (Imanishi et al. 2014). Even if dogs do not display symptoms of infection, they can carry the bacterium for several months and spread it to other animals. Humans can contract salmonellosis from handling contaminated pet foods or fecal eliminations from pets that have ingested contaminated food (CDC 2018). The Food and Drug Administration (FDA) archive of recalls for 2018 lists 17 pet food products that were removed from the market due to potential or confirmed *Salmonella* contamination (FDA 2018c). *Salmonella* in pet foods is ongoing problem, with outbreaks of salmonellosis occurring within the past 5 years with a country-wide outbreak occurring in 2012 that affected over 50 people. For example, 16 brands of dry dog food were recalled in 2012 after 53 humans and 31 dogs across 20 U.S. states were diagnosed with salmonellosis after consuming or handling the pet food (Imanishi et al. 2014). The cause of the outbreak was found to be *Salmonella* Infantis, which was isolated from an asymptomatic dog and traced back to an unopened bag of dog food.

The ability to test dog food products for *Salmonella* quickly and efficiently is essential to preventing salmonellosis in dogs and their handlers. Rapid testing allows for prompt detection and removal of contaminated foods from the marketplace as well as identification of the causative agent in outbreak investigations. Furthermore, rapid testing methods can help reduce the financial loss associated with recalls in an industry that generates approximately $21 billion in dog food sales annually (APPA 2018). Traditional culture-based methods for *Salmonella* testing are effective; however, these methods are time-consuming, generally requiring at least 5 days for confirmed results (Andrews et al. 2006). Real-time polymerase chain reaction (PCR) is a commonly used method for rapid detection of foodborne pathogens (Margot et al. 2013). Real-time PCR can reduce the time to detection to 1-2 days and is sensitive to low levels of bacteria.

However, it is susceptible to inhibitors found in food products, such as polyphenolic compounds (Margot et al. 2013).

Loop-mediated isothermal amplification coupled with bioluminescent assay in real-time (LAMP-BART) is a novel method for pathogen detection that combines isothermal DNA amplification with bioluminescence detection (Gandelman et al. 2010; Yang et al. 2016). Isothermal DNA amplification does not require thermal cycling and has shown greater tolerance to assay inhibitors compared to PCR (Yang et al. 2014; Wang et al. 2008). When coupled with bioluminescence detection, results can be obtained in real-time with a run time of 75 min. In previous studies, LAMP and LAMP-BART were reported to be precise, rapid, and sensitive for the detection of S*almonella* in food products (Yang et al. 2016; Wang et al. 2008). For example, Yang et al. (2016) reported the ability to detect *Salmonella* Infantis in dry dog food at concentrations of 10^0-10^1 CFU/25 when a 24-h enrichment step was used in combination with LAMP-BART. In the absence of the enrichment step, the detection limit was reported to be 10^5-10^6 CFU/25 g.

A potential means for shortening the enrichment period for the detection of low concentrations of *Salmonella* is with microplate immunocapture (IC). Microplate IC utilizes an antibody-coated microtiter plate to concentrate bacterial cells for greater detection efficiency (Arbault et al. 2014ab; Fakruddin et al. 2017; Rogers et al. 2018). Although microplate IC is not as widely used as immunomagnetic separation, it is considerably less expensive because it does not require production of antibody-coated beads. Microplate IC has been used in previous studies to concentrate bacterial cells for detection with PCR and/or selective plating (Rogers et al. 2017; Fakruddin et al. 2017). For example, Fakruddin et al. (2017) found that coupling microplate IC with PCR allowed for detection of *Salmonella* Typhi in 62.7% of food samples inoculated with

concentrations of 10^1-10^5 CFU/25 g, as compared to 56% detection for samples that underwent traditional enrichment plus PCR. Rogers et al. (2018) found that microplate IC coupled with PCR could detect *L. monocytogenes* at levels of 10^0, 10^2, and 10^4 CFU/25g at rates of 88.9%, 94.4%, and 100% respectively, but microplate IC with selective plating yielded 0% recovery at 10^0 CFU/25g and 44.4% at 10^2 CFU/25g. Rogers et al. (2018) did not use a pre-enrichment step prior to conducting microplate IC and selective plating which could explain the limited recovery of bacteria at low concentrations.

The overall objective of this study was to evaluate the use of microplate IC to reduce the enrichment time required for detection of *Salmonella* in pet food with LAMP-BART and selective plating. The specific aims of this study were to: 1) optimize the microplate IC parameters to enable detection of *Salmonella* in dog food and treats within 1 working day (8 h) when combined with LAMP-BART, 2) determine the ability of microplate IC combined with LAMP-BART or selective plating to consistently detect low levels of *S*. infantis (10^0-10^4 CFU/25 g) in dog food and treats, and 3) compare the performance of microplate IC + LAMP-BART, 24-h enrichment + LAMP-BART, microplate IC + selective plating, and 24-h enrichment + selective plating.

2. Review of Literature

2.1 Salmonellosis in humans

In the United States alone, 48 million instances of foodborne illness are reported annually, which are responsible for over 100,000 hospitalizations and an average of 3,000 deaths. Of the 16 organisms responsible for most outbreaks of foodborne illness, *Salmonella* is the most frequently reported bacterial cause and is responsible for up to 4 million cases of salmonellosis annually (FDA 2018a). As of 2007, there were 2,579 known serotypes of

Salmonella, with the two most common types responsible for causing infections in humans being *Salmonella* Enteritidis and *Salmonella* Typhimurium (FDA 2012; USDA 2016). Both serotypes belong to the species *S. enterica*: one of the two species causing illness in humans. *S. bongori* is the other species and can also cause salmonellosis but is not as common (FDA 2012). After ingesting contaminated food products or handling contaminated pet foods, symptoms of salmonellosis occur within 6-48 hours and generally last 4-7 days depending on the serotype and type of illness. Symptoms of salmonellosis generally include intestinal upset, vomiting, stomach cramps and fever (FDA 2018a). It is important that *Salmonella* contamination is detected before it causes infection, and one way to accomplish this is by decreasing the time required for detection in foods.

2.2 *Salmonella* in the food industry

Of the 48 million annual cases of foodborne illness in the United States, *Salmonella* is the cause of 35% of hospitalizations due to illness requiring medical attention (FDA 2018a; Scallan et al. 2011). Reported *Salmonella* infection is most commonly associated with eggs, poultry and meat, unpasteurized milk and cheese, juices, and fruits and vegetables, with animals and livestock being the main sources of the bacteria (FDA 2018a). Because of the ability for *Salmonella* to survive in many different food types, it is a serious problem for all sectors of food processing and manufacturing. The Federal Food, Drug, and Cosmetic Act (FD&C Act) states that any food that contains contaminants that are injurious to health are considered adulterated and are not suitable to be produced and sold to customers (Andrews et al. 2016). The FDA also considers *Salmonella* a zero-tolerance organism in ready-to-eat (RTE) foods, including dog food and treats, so no level of the bacteria is acceptable in foods within those categories (FDA 2017). Therefore, food companies are held to high standards to limit contamination of *Salmonella* in

their processing facilities. By enforcing proper sanitation, thorough quality checks, and routine testing of samples, facilities can limit the spread of bacterial contamination during the manufacturing process. If a contaminated food product is distributed, the company must recall the potentially dangerous products and stop production until they are clear of contamination according to standards set by the FDA and USDA. According to the FDA (2009), the average cost of a food recall is $10 million from loss of product, labor, and potential lawsuit settlements, but is also costly in terms of reputation. Given the high costs of *Salmonella* outbreaks, methods of limiting contamination are very important.

While the standards for food products differ depending on processing procedures and the type of food, the FDA outlines many methods to reduce the risk of S*almonella* contamination in human-foods and direct-human-contact to animal foods (foods that humans handle to feed to pets or animals). The testing procedures do not pertain solely to processing plants, but are also applicable to any facilities that handle, pack, hold, or transport the food from initial ingredients to the final product. The FDA's Bacteriological Analytical Manual (BAM) outlines several culture methods for the detection of *Salmonella*. Companies can include one of these testing methods or an FDA approved non-BAM method in their HACCP (Andrews et al. 2016). In addition to defining specific methods, the manual also includes precise directions for handling the food products during testing in order to limit spread of *Salmonella* through cross-contamination with other products, or transfer from a handler to other places in the processing facility. *Salmonella* testing methods can yield one of three results: a presumptive positive, which requires more testing; a confirmed positive, which requires cessation in production and destruction or reconstitution of the batch; or a confirmed negative. No matter the outcome of the initial testing, multiple repetitions from multiple representatives of a production batch are

required to ensure the product is free of *Salmonella* (Andrews et al. 2016). BAM is revised yearly based on new developments in testing methods and new serotypes associated with *Salmonella* contamination, and enhanced methods are included if found to be more reliable and specific than traditional methods.

2.3 *Salmonella* in pet foods

Salmonella is not only a concern for human products, but also for pet foods. Pet foods are often comprised of ingredients sourced from meat and poultry, which are major sources of *Salmonella* contamination (USDA 2016; Huang et al. 2009). While the USDA is responsible for regulating most meat products, the FDA regulates pet food products. It is important to note that the FDA does not consider animal feed and pet food as the same. Animal feed is intended for animals that are used in the human food supply, and pet foods are canned and bagged food and treats for common household pets (FDA 2018b). Pet foods are regulated by FDA according to the FD&C and manufacturers must follow guidelines like those established for human foods. This includes the requirements that pet food go through a 5-log reduction during processing, be processed in sanitary conditions, and be truthfully labeled (FDA 2018b; Zicker 2008). However, the FDA does not require pre-market approval of pet foods as long as food ingredients, colorings, fortification agents, and preservatives are generally ruled as safe (GRAS). Guidelines for labeling pet foods are also outlined by the Association of American Feed Control Officials (AAFCO) and include: listing ingredients by weight, statement of identity (SOI), location and name of the distributor, and net quantity.

Salmonella can contaminate the starting raw ingredients used in pet food and there are several avenues by which *Salmonella* can be introduced during processing, as described below. In 2018, there were 17 recalls of pet food caused by *Salmonella*. The amount of *Salmonella*-

related recalls in 2018 was higher than previous years, with 8 total recalls of pet food from January 2016-December, 2017. Another problem associated with the introduction of *Salmonella* to pet food ingredients can be traced to the source of the meat ingredients. Despite the guidelines set out by the FDA, human foods being tested for *Salmonella* that are deemed to be adulterated can be used for another feed source if it is not direct-human-contact to animal (Andrews et al. 2016).

In 2018, dog food recalls due to *Salmonella* were most associated with raw pet foods as opposed to canned or otherwise processed foods. However, *Salmonella* can contaminate freeze-dried, frozen, extruded, and canned dog food products. When considering all recalls from January 2016-December 2018, 15/25 recalled pet food products were non-raw food products. While salmonellosis is dangerous to pets, humans can also contract *Salmonella* from their dog or simply from handling contaminated dog food (CDC 2013). The zoonotic nature of salmonellosis further increases the need to make pet foods as safe as foods for human consumption.

2.4 Occurrence of *Salmonella* in dog food and treats

All dog food products are at risk for *Salmonella* contamination, but most recent outbreaks have been associated with raw dog food products (FDA 2018c). Wet dog food is often preserved through canning which sterilizes the product. Raw meat dog food applies freezing or refrigeration as defenses against bacteria, but changes in temperature and freeze-thaw cycles can create lead to more suitable environments for *Salmonella* to thrive (Zicker 2008). Although Salmonella prefers an A_w of 0.94 or greater, dry dog food is a low moisture food that can support survival of the bacteria as some serotypes are semi-resistant to the drying process and can survive desiccation conditions, as seen in recent *Salmonella* outbreaks in spices and other dry products (FDA 2015; Lambertini et al. 2016). As dry dog foods come in large units that are not

typically used in one meal, the package is left open and stored in ambient temperatures where Salmonella can survive until exposed to an environment suitable for growth (Lambertini et al. 2016). The temperature and other environmental conditions favored by *Salmonella* are shown in Table 1.

Table 1. Optimal conditions for *Salmonella* growth with examples*

Parameters	Growth conditions	Examples of foods
Acidity	pH 4.2 or greater	Potatoes, spinach, bread, flour, meat, fish, eggs
Time	Doubling time of 2 h above 21°C	Any susceptible foods from processing to sale
Temperature	5-47°C, optimum 37°C	Refrigerated meats, milk, produce. Flour, breads, or snack foods at ambient temperatures
Oxygen	Facultative anaerobe	Any
Moisture	A_w>0.94	Liver, cheese spreads, fresh meat, bread

*(USDA 2012; Lawley 2013; FDA 2015)

There have been a couple of outbreaks of *Salmonella* associated with pet food products over the past decade. In 2016, an unspecified strain of *Salmonella* led to a recall of a contaminated frozen beef pet food product from 6 states after reports of 2 sick cats and one puppy fatality (FDA 2018C). A more devastating recall occurred in 2012 when Diamond® pet foods recalled 16 brands of their dry dog food after 53 humans and 31 dogs over 20 states were diagnosed with salmonellosis after handling the pet food. No deaths were reported in association with the 2012 outbreak, but 10 of the 53 people required hospitalization, and *Salmonella* was found in asymptomatic dogs that had consumed the contaminated product (Imanishi et al. 2014).

The 2012 outbreak was traced back to a bag of unopened dog food from Diamond® pet foods, and the strain was isolated from an asymptomatic dog that had consumed that brand of

food (Imanishi et al. 2013). The strain that caused the outbreak was *S. enterica* serotype Infantis. A survey conducted between 2002-2009 isolated a total of 45 different *Salmonella* serotypes that contaminated pet food and treats and animal feed, with *S.* Montevideo and *S.* Senftenberg being the most commonly isolated serotypes (Li et al. 2012). There are over 2,500 known serotypes of *S. enterica* associated with illness in humans, but only a fraction of these have been isolated from dog food alone. The 5 major serotypes associated with human illness are Newport, Enteritiditis, Typhimurium, Heidelberg, and Hadar. These serotypes are commonly found in meat and poultry, ingredients which commonly supply ingredients for dog food (FDA 2012; Lambertini et al. 2016; Bugarel et al. 2017). Li et al. (2012) found *S. enterica* in 12.5% of samples pulled from commercially available dog foods.

2.4.1 Composition of dry dog food

Dog food makes up the greatest sector of the pet food industry, accounting for approximately $21 billion in annual sales out of a total of $29 billion for pet food overall (FDA 2018C; Zicker 2008; APPA 2018). Dry dog food, commonly referred to as kibble, is a low-moisture food that does not require refrigeration or cooking and can support survival of *Salmonella* (FDA 2012). The matrices and ingredient composition of dry dog food and treats provide a suitable environment for *Salmonella* to survive. Additionally, with the lipid coating commonly applied to the food after thermal processing, *Salmonella* can be protected within the food, or on the surface as it is can be introduced with the addition of fat (Lambertini et al. 2016).

The matrices of dry dog foods are commonly composed of proteins, carbohydrates, and fats. The net weight of ingredients used in kibble is mainly some type of grain or reconstituted animal feed, followed by meat, animal or vegetable fats, flavorings, and preservatives (Zicker 2008). Major dog food brands typically include numerous grains and meals as major ingredient

sources. A typical ingredient label is shown in Figure 1**.**

Ingredients

Chicken by-product meal, wheat flour, whole grain ground corn, rice bran, dried beet pulp, chicken fat (preserved with mixed tocopherols), millet, ground white rice, fish meal, egg product, flaxseed, natural chicken flavor, potassium chloride, salt, choline chloride, vitamin E supplement, iron proteinate, zinc proteinate, copper proteinate, ferrous sulfate, zinc sulfate, copper sulfate, potassium iodide, thiamine mononitrate, manganese proteinate, manganese oxide, ascorbic acid, vitamin A supplement, biotin, niacin, calcium, pantothenate, manganese sulfate, sodium selenite, pyridoxine, hydrochloride (vitamin B6), vitamin B12 supplement, riboflavin, vitamin D supplement, folic acid.

Figure 1. Ingredient statement from Diamond® pet foods Maintenance dry dog food. Animal and grain-based ingredients are underlined (Adapted from: Diamond, 2017)

While animal products are most commonly associated with *Salmonella* contamination, the high amount of grains that comprise dry dog foods are sources of *Salmonella* as well. Once harvested or reconstituted, the grains and cereals are milled and crushed, and then sent to manufacturers for use in other products. It is not required for the grains to be heat treated before going to another processing plant, so *Salmonella* from the original source can cross-contaminate other products during transport, storage, and processing. Additionally, because the origin feed or grains are crushed and milled at the source, the *Salmonella* count can be confined to a small section of the feed and are not representative of the entire batch if only clean samples are pulled and tested (Davies and Wales 2013).

While carbohydrates are a major source of concern in dry dog foods, the fat components also present problems for detecting *Salmonella.* The ingredient statement in Figure 1 lists chicken meal and chicken fat within the first 6 ingredients. The major *Salmonella* serotypes

associated with dog foods are most widely found in poultry products, again causing greater risk of contamination in chicken-based dog foods, or chicken meal-based foods (Huang et al. 2009). Once the grains and meals are mixed together, they are cut into kibble, dried by heating, and then coated in either animal or plant fats. Lambertini et al. (2016) suggest the fat on the outside of the kibble pieces provides a protective layer to bacterial cells that survive the extrusion process or contact the food product between drying and coating. The lipid layer can also protect *Salmonella* on the interior layers of the dry kibble or on the outside of the final product prior to packaging (Lambertini et al. 2016). *S. enterica* is not only a problem pertaining to animal fats, but brands that rely on vegetarian formulations and use vegetable glycerin or other types of plant fat as coating can come from farms contaminated with *Salmonella* as well (FDA 2012).

2.4.2 Challenges for *S. Enterica* contamination in dry dog foods

In addition to the matrices of dry dog food that cause *Salmonella* concerns, processing also may introduce *Salmonella* into dog food. Most dry dog foods are processed the same way. First the individual ingredients are mixed together and then extruded to create long strands of dog food of a certain diameter. As the food passes through the extruder, steam, heat, and pressure are applied to reduce microbial loads to commercially safe levels of a 5-log reduction for *Salmonella*, and the strands are cut into the desired size of the kibble (Zicker 2008). The pieces are then dried until they reach a low water activity (a_w) of 0.1 and then are finally coated in fats and flavoring before being packaged (Lambertini et al. 2016).

The combination of steam, heat, and pressure applied during extrusion is enough to reduce microbial loads in the dog food and destroy *Salmonella*, but mechanical error or resistant serotypes can lead to contamination post-extrusion. The extrusion temperature usually processes the food between 100-200° C to effectively destroy *Salmonella,* but this is not the last step in

manufacturing dry dog foods (Zicker 2008). After extrusion, the kibble is dried and then coated with fat and any additional preservatives and flavorings. If *Salmonella* has contaminated the drying equipment during the intermediate processing stage, then the layer of fat can protect the bacteria. Additionally, no pathogen reducing steps are applied to the kibble after addition of the fats, so *Salmonella* can be presented in the final product as well (Lambertini et al. 2016).

The next problem is the 0.1 a_w required for dry dog foods. Despite the a_w of 0.94 or greater favored by *Salmonella* as seen in Table 1, several serotypes of *S.enterica* have been found to develop tolerances to desiccation (Gruzdev et al. 2011). After extrusion, the kibble is dried from an a_w of 0.25 or greater to 0.1 (Zicker 2008). Ideally there should be little to no *Salmonella* present after the heat treatment, but Gruzdev et al. (2011) found 4 serotypes of *S. enterica* with tolerance to temperatures close to 100°C in conjunction with a preference for low-moisture environments. The serotypes: Typhimurium, Enteritidis, Newport, and Infantis were all tolerant to high heat, ethanol, citric acid, and dehydration, or a combination of multiple processing hurdles. Other possible forms of survival through the extrusion process can potentially be attributed to cross-contamination from humans or machine dysfunction, but the resi ence of these serotypes to multiple processing steps causes new problems for the pet food industry.

Along with tolerance to standard processing conditions, *S. enterica* can survive in dry conditions for a long time. Lambertini et al. (2016) studied plate counts of *Salmonella* in dry dog food when stored at ambient temperatures during processing and factory and household storage. Processed dry dog foods typically have a year-long shelf life, but Lambertini et al. (2016) detected *Salmonella* in pet foods up to 19 months post-processing, showing that the bacteria can survive even longer than the pet food product is in the market or kept in storage. While the

bacteria did show a rapid log reduction after three months of storage at ambient temperatures, it leveled off after the initial three months and was still measurable and quantifiable up to 7 months past the intended shelf life. This shows that dry dog food that may go unopened for several months in storage at ambient temperatures may contain *Salmonella* that can be present in multiple units from the same processing batch.

2.5 Conventional methods for detection of *Salmonella*

With *Salmonella* being a major cause of foodborne illness, it is important to test for its presence at several steps during processing. Sample testing is critical for quality assurance departments, but the methods employed are not always the most effective or efficient. Once a sample is pulled from the raw materials, product batch, or finished, non-packaged product, the food needs to be prepared for bacterial isolation, enriched, cultured, and plated. These steps are very time consuming, and conventional methods can take up to 4-6 working days to get a confirmed result (FDA 2012; Margot and other 2013). The medium and reagents for testing change depending on the matrices and ingredients of the food product, so companies that produce several different types of pet foods may need to employ several different culturing methods which can be costly due to the reagents and equipment. A process diagram for testing dry dog food for *Salmonella* with a conventional culture method can be seen in Figure 2.

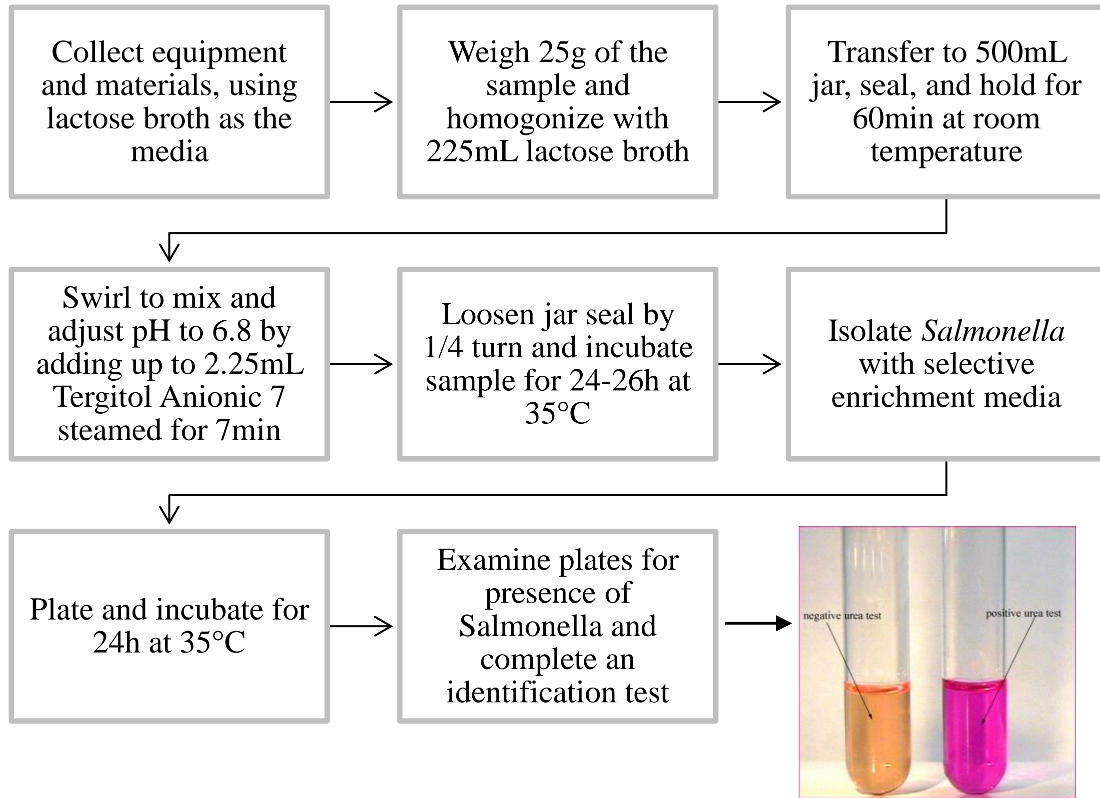

Figure 2. process diagram of a traditional cultural method for *Salmonella* Typhimurim in animal food products with a pure culture and urease test. A positive result of *Salmonella* is signified by the color pink (Andrews et al. 2016; Aryal 2015)

Figure 2 does not highlight the time required to obtain a confirmed result, nor exemplify the repetitions of each test. For *Salmonella* testing alone, BAM lists 26 pieces of necessary laboratory equipment (including everything from beakers to an autoclave) and 54 types of media used for conventional methods, which small companies with limited space or funds may not have access to. The different media each pertain to 1 of 27 defined food types, and once the samples are prepared and incubated for the first 24 h, isolating the *Salmonella* strain depends on the specific serotype of concern for that company and food. Identifying the specific *Salmonella* strain also affects the culture used, and whether it should be mixed, pure, flagellar, or serological. If the *Salmonella* serotype happens to be urease negative, then there are 6 other cultural methods

that can be appropriate for the sample, again dependent on the type of food (Andrews et al. 2016). While conventional detection methods are the "gold standard" for newer methods of *Salmonella* detection, selecting the correct testing method for each food type and serotype is complicated for foods with many ingredients, and laborious compared to methods that allow for confirmed results in less than the average 5 days of these traditional methods (Nemser et al. 2014).

2.6 Emerging methods for rapid detection of *Salmonella*

Conventional testing methods are multifaceted and time intensive. High-volume manufacturers that produce tons of dry dog food per day may not have time to test representative samples of all the products throughout the facility. Smaller-volume companies conversely may not have the money or equipment for testing different product types. Since maintaining a safe food supply is imperative, the need for rapid detection of *Salmonella* led to development of new methods that shorten the testing period to 1-2 days as compared to 4-6 days with conventional methods. One rapid method commonly used for detection of *Salmonella* in the food industry is real-time PCR (FDA 2012). Like conventional cultural methods, real-time PCR follows a series of steps but it is more expedited as it uses DNA replication to target *Salmonella.* First the food samples are prepared, inoculated and enriched. After the enrichment, the samples are incubated for 16-24 h, and then the samples are plated to obtain a concentration of the bacteria which is replicated by the addition of a DNA polymerase and heat denaturation from thermal cycles (Wang et al. 2008). Afterwards, the DNA is extracted and compared to the DNA of specified *Salmonella* serotypes (Margot et al. 2013). This is a very simplified overview of real-time PCR, and the assay that is used changes the enrichment period and incubation time. Table 2 shows testing parameters for a common real-time PCR method for *invA*, a gene found in all *Salmonella*

isolates from human and pet foods.

Table 2. Comparison of 3 methods for detecting the *invA* gene in *Salmonella* serotypes*

	Real-Time PCR	**LAMP**	**LAMP-BART**
***Salmonella* Serotype**	*S*. Choleraesuis	*S*. Choleraesuis	151 different serovars
DNA polymerase	*Taq*	Strand displacing *BST*	Strand displacing *BST*
Temperature (°C)	30 cycles at 94°C for 30s, 55°C for 30s, 72°C for 30s, and 72°C for 7min	60-65°C	60°C
Reaction Time (min)	260	60	75
Specificity (%)	96	100	100

*Adapted from (Wang et al. 2008; Yang et al. 2016)

While real-time PCR reduces the time needed to obtain presumptive results, it still requires a culture to confirm the presence of *Salmonella*. Additionally, the matrices of some dog foods can reduce sensitivity of certain real-time PCR tests to multiple serotypes or limited presence of the bacteria (Margot et al. 2013). Real-time PCR is a rapid detection method compared to conventional culture methods. However, due to the need for enrichment, it can still take up to 2 days to confirm the presence of *Salmonella*. To address the problem of time required to confidently detect the presence of *Salmonella* in foods, new technologies and methods that allow for confirmation in less than 2 days with the same effectiveness of conventional methods and PCR are being explored (Wang et al. 2008; Yang et al. 2016).

2.6.1 Loop-mediated isothermal amplification with bioluminescent assay in real-time (LAMP-BART)

Coupling loop-mediated isothermal amplification with bioluminescent assay in real-time (LAMP-BART) can reduced the time needed for *Salmonella* detection (Yang et al. 2016). LAMP-BART, like PCR, is a DNA-based method that amplifies target DNA, but does so in a shorter time (Yang et al. 2016). Notomi et al. (2000) developed a LAMP method to amplify DNA with increased sensitivity and robustness compared to cultural methods or PCR. The method uses a strand-displacing DNA polymerase, *Bst,* and 4 designed primers to detect target DNA at 6 distinct sequences. The innermost primer contains sequences of the *Salmonella* DNA to initiate LAMP, and then the resulting single strand of copied DNA serves as another primer that hybridizes the inner and outer primers to create stem-loop DNA. This autocycle continues until 10^9 copies of the target *Salmonella* DNA have formed, all of which takes place in under an hour (Wang et al. 2008; Yang et al. 2016). LAMP is an isothermal method and relies on the strand-displacing polymerase as opposed to heat denaturation of PCR to create a single strand DNA template (Gandelman et al. 2010). Additionally, LAMP is less influenced by non-target DNA than PCR which increases the effectiveness of LAMP (Notomi et al. 2000).

While being highly sensitive and efficient, there are some limitations to isothermal nucleic acid amplification technologies (iNAATs) like LAMP, such as the assay they employ to detect target DNA. Methods like real-time PCR that use the synthesis of polynucleotides of target DNA to detect *Salmonella* are limited by the costly and sensitive fluorescence technology that rely on emitted light from the polynucleotides. To address the problems associated with real-time detection of pathogens, the novel Bioluminescent Assay in Real Time (BART) can be used to measure the production of inorganic pyrophosphate (PP_i) which is proportional to the number

of polynucleotides amplified during DNA replication. LAMP produces a high number of PP_i which is converted to ATP for bioluminescent reading. BART quantifies the PP_i produced during LAMP by using an assay of firefly luciferase to emit light as it is transferred to ATP (Gandelman et al. 2010). Real-time PCR utilizes costly equipment that is sensitive to non-target DNA and requires proper thermal cycling, but LAMP-BART enhances the method and allows for real-time detection with the use of a simple, portable light detector as seen in Figure 3 (Yang et al. 2016).

LAMP-BART is advantageous over PCR because the latter uses the *Taq* DNA polymerase which is sensitive to assay inhibitors such as reagents or non-target DNA that make it less sensitive to target DNA (Yang et al. 2016). LAMP uses a strand-displacing *Bst* polymerase which is not sensitive to inhibitors and results in greater specificity which is preferable for the foods, like dry dog food, with more complex matrices (Yang et al. 2014). PCR primers must be selected specifically for the type of food that is being tested and that can also apply the *Taq* polymerase, but the 4-6 primers used in conjunction with *Bst* polymerase for LAMP have a wider application and are not dependent on the matrices of the food product. LAMP is also preferable over PCR as it is conducted in an isothermal environment at a constant temperature of 65°C which does not require thermal cycling (Wang et al. 2008). While both PCR and LAMP are sensitive methods, LAMP-BART is time and energy efficient, and requires less target DNA to determine the presence of *Salmonella*. Refer to Table 2 for a comparison between real-time PCR, LAMP, and LAMP-BART.

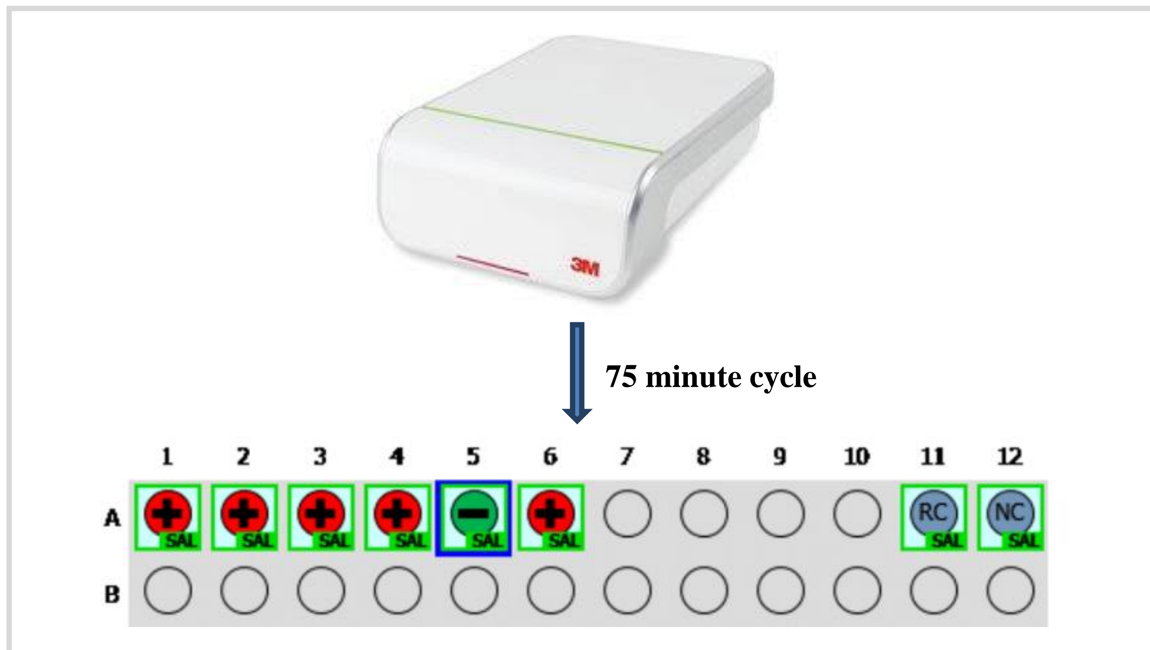

Figure 3. A 3M Molecular Detection instrument for LAMP-BART, and an example of the simple computer-generated results. A red positive symbol confirms presence of *Salmonella*, and a green negative symbol confirms absence (3M, 2018)

2.6.2 Microplate immunocapture

When looking to detect low levels of *Salmonella* (10^0-10^1 CFU/25g), LAMP-BART still requires a 24 h enrichment period when detecting low levels of the bacteria ($\leq 10^4$ CFU/25g) (Yang et al. 2016). Immuno-concentration methods can be used to shorten the enrichment time by concentrating the target pathogen . Hara-Kudo et al. (2001) examined the use of the Vitek immuno diagnostic assay system (VIDAS-ICS) and immunomagnetic separation with immunomagnetic beads to detect *S. enteritidis* in contaminated shell and liquid eggs respectively. VIDAS-ICS and selective plating on 8 different media agars allowed detection of the bacteria in all 12 shell egg samples. Immunomagnetic separation with liquid eggs also had detection of the bacteria in all samples with selective plating on 4 different media types, with contamination levels as low as 1 CFU/25g.

Immuno-concentration has not been coupled with LAMP-BART for detection of *Salmonella* in food, but previous research on combining microplate immunocapture (IC) with PCR found it to be more efficient and sensitive in *Salmonella* detection than PCR alone. Arbault et al. (2014) found that when testing for *E. coli* with using microplate IC, $5.4*10^5$ CFU was recovered in ground beef as opposed to only $2.3*10^3$ CFU on the same sample using a traditional method. Although the authors were not specifically isolating *Salmonella*, the results indicate potential use of this method for detecting other bacteria.

Rogers et al. (2018) found that microplate IC coupled with PCR could detect *L. monocytogenes* at levels of 10^0, 10^2, and 10^4 CFU/25g at rates of 88.9%, 94.4%, and 100%, respectively, but microplate IC with selective plating yielded 0% recovery at 10^0 CFU/25g and 44.4% at 10^2 CFU/25g. Fakruddin et al. (2017) found that coupling microplate IC with PCR allowed for detection of *Salmonella* Typhi in 62.7% of food samples inoculated with concentrations of 10^1-10^5 CFU/25 g, as compared to 56% detection for samples that underwent traditional enrichment plus PCR. Neither study used a pre-enrichment step prior to conducting microplate IC and selective plating

2.7 Rationale and significance

Determining a method for rapid detection of *Salmonella* in dry dog foods that is quick, reliable, and robust is important to facilitate sample testing and reduce the number of contaminated products on the market. *Salmonella* is one of the leading causes of foodborne illness in the United States and is responsible for the most annual hospitalizations and deaths from microbial contamination of food. Because *Salmonella* is zoonotic, it can be spread between animals and humans, so the safety for both human foods and animal feed are equally as important. Dry pet foods are regulated by the FDA and have complex matrices that support

survival of *Salmonella,* which is resistant to low moisture content. Due to processing conditions, the formulation of dry dog foods and treats, and the ability of *Salmonella* to survive in dog food, the risk of contamination is high. Traditional culture methods take 4-6 days to positively confirm the presence of *Salmonella* in dog food, which is time consuming and laborious for high-volume manufacturers. Rapid detection methods that take less than 24 h to confirm the presence of *Salmonella* will benefit the pet food industry by allowing for more samples to be tested at a greater speed. Microplate immunocapture combined with LAMP-BART is a possible way to shorten the enrichment period and detection method to achieve results that are both more sensitive and robust than traditional methods or LAMP-BART alone. The two objectives of this study were to 1) optimize the microplate IC parameters to enable detection of *Salmonella* in dog food and treats within 1 working day (8 h) when combined with LAMP-BART, 2) determine the ability of microplate IC combined with LAMP-BART or selective plating to consistently detect low levels of *S*. infantis (10^0-10^4 CFU/25 g) in dog food and treats, and 3) compare the performance of microplate IC + LAMP-BART, 24-h enrichment + LAMP-BART, microplate IC + selective plating, and 24-h enrichment + selective plating.

There have been no studies into LAMP-BART combined with microplate immunocapture. This research aimed to contribute to optimization of the best method of Salmonella detection by comparing LAMP-BART with traditional enrichment to LAMP-BART with microplate immunocapture on a sample of dry dog food.

3. Materials and Methods

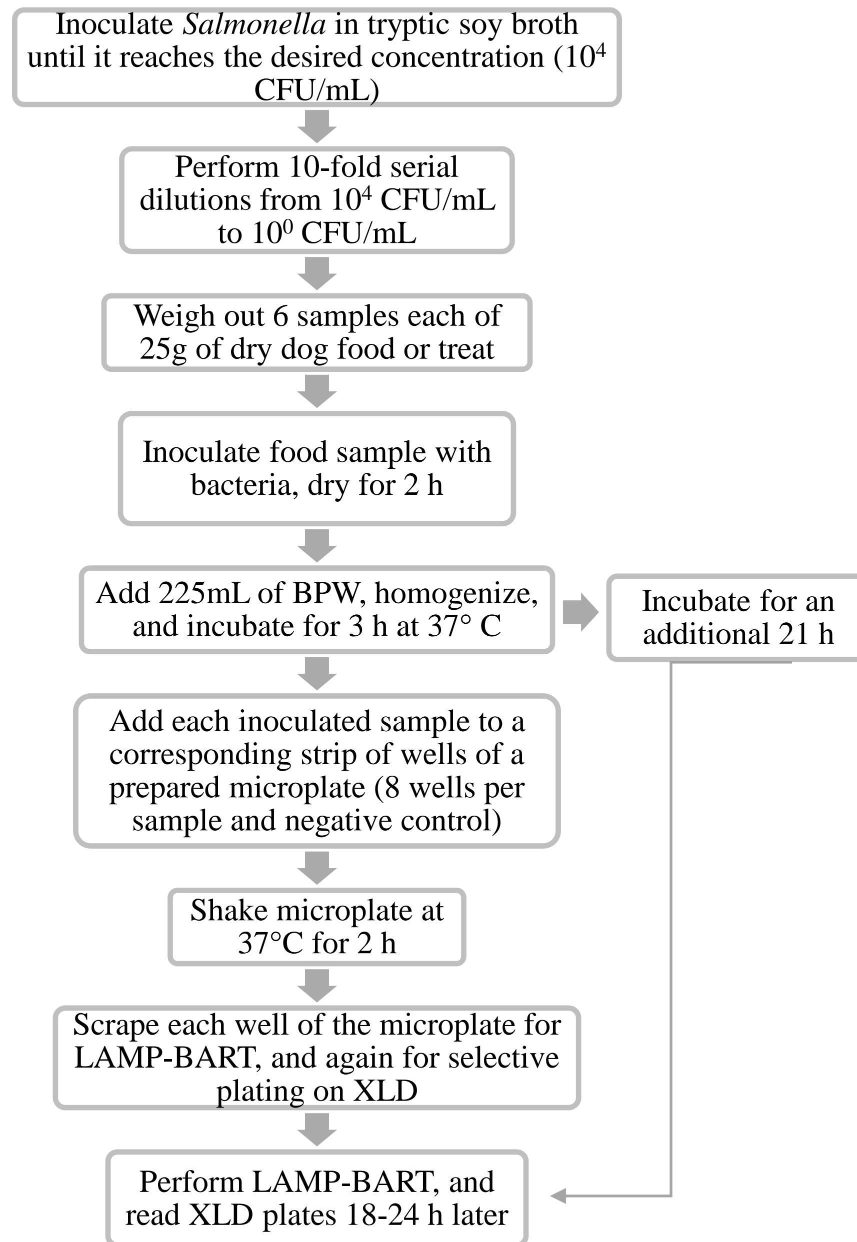

Figure 4. Experimental design repeated in triplicate for TSB, dog food, and dog treats

3.1 Bacterial isolation and preparation

Salmonella enterica serovar Infantis ATCC® 51741 was obtained from American Type Culture Collection® [(ATCC) (Manassas, VA)]. All media used in this study were from Becton, Dickinson and Company [(BD) (Franklin Lakes, NJ)], unless otherwise specified. Bacterial isolation was conducted by streaking the stock culture of *S*. Infantis onto tryptic soy agar (TSA) and incubating for 48 h at 37°C. An isolated colony was transferred from TSA to 10 mL of tryptic soy broth (TSB) and incubated at 37°C until the bacteria reached the desired concentration of 10^4 colony-forming units (CFU)/mL.

3.2 Microplate preparation

Salmonella Polyclonal Antibody PA1-7244 (Invitrogen™, Carlsbad, CA) was diluted to 1 µg/mL in carbonate-bicarbonate buffer, pH 9.6. The microplate was prepared according to a protocol adapted from Abcam (http://www.abcam.com/protocols/sandwich-elisa-protocol-1). First, 200 µL of the antibody solution were added to individual wells of a 96-well polystyrene microtiter microplate separated into 8-well strips. The plate was covered with sterile polyethylene sealing films (Excel Scientific, Victorville, CA) and incubated at 4°C for 24 ± 2 h. Plates that were not used immediately were stored at -20°C with the antibodies in each well, and then prepared according to the following procedure. The wells were washed twice with 200 µL 1X phosphate buffered saline [(PBS) (Fisher Scientific, Hampton, NH)]. Next, 200 µL of 5% non-fat dry milk prepared in 1X PBS was added to each well and incubated for 2 h at room temperature. Immediately before performing microplate IC, the blocking agent was removed, and the wells were washed two times with 200 µL 1X PBS.

3.3 Microplate IC for broth samples

The experimental conditions for microplate IC of *S.* Infantis were first optimized in the absence of a food matrix. The goal of optimization was to determine the microplate IC conditions that would allow detection of *S.* Infantis at concentrations of 10^0 CFU/mL using selective plating on XLD. The bacterial culture was prepared as described above, followed by serial dilution in TSB to allow for concentrations of 10^0 to 10^4 CFU/ml. Then, 1.6 mL of each dilution was distributed across an 8-well strip of the prepared microplate for a total of 200 µL of sample per well. An un-inoculated broth sample was used as a negative control. The microplates were incubated using an Eppendorf ThermoMixer® C (Hamburg, Germany) with a shaking speed of 300 rpm. The procedure was optimized for incubation temperature (23°C vs. 37°C), number of fill cycles (1-4), and cycle incubation time (15-60 min). Each fill cycle involved addition of 1.6 mL of the sample to the corresponding 8-well microplate strip, followed by incubation and shaking on the Thermomixer for a given cycle incubation time. The liquid portion of the sample was removed from the microplate and discarded in between fill cycles using a multi-channel pipette.

Following microplate IC, the 8 wells corresponding to each sample and negative control were scraped with a sterile inoculating loop and streaked onto xylose lysine deoxycholate (XLD) agar. The plates were incubated at 37°C for 18-24 h and then examined for typical *Salmonella* growth. The optimized microplate IC conditions (Table 3) were tested in three independent trials with both XLD agar and LAMP-BART (described in section 3.4).

Table 3. Optimized protocol for microplate IC for TSB and food samples
.

Matrix	Pre-enrichment time (h)	Micro-plate incubation time (h)	Microplate incubation temperature (°C)	Plate shaker speed (rpm)	Number of fill cycles	Microplate wells used	Time to detection (h)[a]	
							LAMP-BART	XLD
TSB	0	2	37	300	1	8	3.75	27
Dry dog food/pig ear treats	3	2	37	300	1	1	6.75	30

[a]Time to detection includes sample preparation (30 min), pre-enrichment (0-3 h), microplate IC (2 h), and LAMP-BART (75 min) or XLD (24 h incubation).

3.4 Microplate IC for food samples

An 11-kg bag of chicken-flavored, dry dog food and a 4.5-kg bag of pig ear dog treats were purchased from a local retail outlet in Orange, CA, USA. The dog food products were confirmed negative for the presence of *Salmonella* according to the conventional culture method described in the Bacteriological Analytical Manual (BAM) (Andrews et al. 2016). A bacterial culture of *S.* Infantis was prepared as described above, followed by serial dilution in buffered-peptone water (BPW). Dog food/treat samples (25 g) were spot-inoculated with *S.* Infantis with concentrations of 10^0 to 10^4 CFU/25g. An un-inoculated sample was used as a negative control for each trial. The samples were dried in sterile plastic bags inside a biosafety cabinet for 2 h at room temperature, then 225 mL of pre-warmed (35°C) BPW was added to each sample. The inoculated dog treats were mixed by swirling and the dry dog food samples were homogenized in a Stomacher 400C (Seward Laboratory Systems Inc., Bohemia, NY) for 2 min at 260 rpm (Yang et al. 2016).

Dog food samples were optimized for pre-enrichment times (0-3 h) at 37°C prior to microplate IC. Optimization for microplate IC was carried out for dog food for the number of fill

cycles (0-4) and cycle incubation times (15-60 min) at 37°C at a speed of 300 rpm. The effectiveness of scraping only 1 well of the microplate for each sample was also compared to the effectiveness of scraping 8 wells. After microplate IC was completed, the well(s) of the microplate were scraped with a sterile inoculating loop and streaked to XLD agar. The plates were examined for typical *Salmonella* growth after incubation for 18-24 h at 37°C. The optimized conditions for dog food were used for pig ear treats without further optimization. The optimized microplate IC conditions (Table 3) were tested in three independent trials with both XLD agar and LAMP-BART (described below) for dog food and treat samples. The inoculated dog food/treat samples were also tested three times using a traditional 24-h enrichment in BPW at 37°C (Yang et al. 2016), with no microplate IC step. After the 24-h enrichment, each sample was streaked onto to XLD agar and tested with LAMP-BART (described in section 3.5).

3.5 LAMP-BART

LAMP-BART was conducted on a Molecular Detection System (3M Food Safety, St. Paul, MN), software version 2.2.0.0, using the 3M Molecular Detection Assay 2 – *Salmonella* kit Protocol 2. Following microplate IC, an inoculating loop was used to scrape the wells of the microplate and transfer bacteria to a sterile tube containing the pre-mixed lysis solution. Eight wells were scraped per sample for the TSB trials, while only 1 well was scraped per sample for the dog food and treat trials (based on the results of microplate IC optimization). Next, 20 µL of the liquid portion of the sample was removed from the microplate and added to the same tube. For the 24-h enrichment samples, 20 µL of the liquid portion of the sample was added to each sample tube. For the negative control and reagent (positive) control for LAMP-BART, 20 µL of sterile, pre-warmed (35°C) BPW was added to the corresponding tubes of lysis solution. All lysis tubes were held in a dry heat block for 15 min at 100°C and then cooled in a chilling block at

ambient temperature for 5 min. Next, 20 µL of each sample or control was transferred to its corresponding reagent tube. The reagent control was provided with the 3M Molecular Detection Assay 2-*Salmonella* kit to serve as a positive control, while the negative control contained only sterile enrichment medium (BPW). The results for each sample were signified by the Molecular Detection System software with an output of either a red positive symbol if *Salmonella* was detected, or a green negative symbol if *Salmonella* was not detected.

4. Results and Discussion

4.1 Microplate IC optimization

The optimized conditions for microplate IC with TSB and food samples are shown in Table 3. During the TSB optimization, it was found that a microplate incubation time of 2 h at 37°C enabled detection of *S.* Infantis on XLD at levels as low as 10^0 CFU/25g. The use of multiple fill cycles was expected to increase the sensitivity of the assay by allowing more bacteria to adhere to the antibodies coated onto microplate wells; however, it was found that just one fill cycle resulted in comparable growth on XLD, in addition to reducing the labor and time needed for microplate IC. During optimization with dog food, short pre-enrichment periods of 1.5 to 3 h at 37°C were examined prior to running microplate IC. A pre-enrichment period of 1.5 h followed by microplate IC resulted in detection of *Salmonella* in dog food as low as 10^1 CFU/25g using XLD agar (data not shown), while a pre-enrichment period of 3 h followed by microplate IC enabled detection of *Salmonella* in dog food at the lowest level tested (10^0 CFU/25). Therefore, a 3-h pre-enrichment step at 37°C prior to microplate IC was incorporated into the workflow for food samples (Table 3).

While the TSB trials were completed using 8 wells of the microplate for each concentration, later optimization with dog food showed that scraping only 1 well of the

microplate resulted in comparable growth on XLD. These results were unexpected, as it was thought that there would be a greater chance of capturing bacteria if more wells were scraped. However, the use of a 3-h pre-enrichment step combined with the 2-h microplate incubation time likely increased the number of bacteria sufficiently to enable detection based on just one microplate well.

4.2 Microplate IC trials

As shown in Table 4, the optimized conditions determined for microplate IC enabled detection of *S.* Infantis in 100% of the TSB samples tested across three trials. All 15 samples were detected with LAMP-BART and confirmed with selective plating on XLD at all concentrations tested (10^0-10^4 CFU/mL). Similarly, *S.* Infantis was detected in 100% (15/15) of dry dog food samples and pig ear treats tested with microplate IC combined with selective plating on XLD, even at the lowest detection level (10^0 CFU/25 g). These results are consistent with those found when the food samples underwent a 24-h enrichment period followed by selective plating on XLD (Table 4), indicating that microplate IC could be used to shorten the time required for confirmation of *Salmonella* using selective plating to 27-30 h as opposed to 48 h using the traditional 24-h enrichment. In contrast to the current study, previous research has reported limited recovery of foodborne pathogens when microplate IC was combined with selective plating (Rogers et al. 2018; Fakruddin et al. 2017). Rogers et al. (2018) achieved 0% recovery of *Listeria* from cheese and milk samples inoculated at 10^0 CFU/25 g, and only 44.4% recovery at 10^2 CFU/25 g. Similarly, Fakruddin et al. (2017) detected *Salmonella* Typhi in only 13.1% of minced beef samples inoculated at a level of 10^1 CFU/25 g. However, neither of the previous studies used a pre-enrichment step prior to conducting microplate IC. The 3-h pre-

enrichment step employed in the current study likely provided sufficient time for *Salmonella* to grow to detectable levels when combined with microplate IC and selective plating on XLD.

Table 4. Detection rates for *S.* Infantis in broth and food samples following microplate IC or a 24-h enrichment step.

Matrix	*Salmonella* Infantis concentration[a]	Rate of detection (no. positive samples/total no. samples)			
		Microplate IC[b] + XLD	24-h enrichment + XLD	Microplate IC + LAMP-BART	24-h enrichment + LAMP-BART
TSB	10^4	3/3	N/A	3/3	N/A
	10^3	3/3	N/A	3/3	N/A
	10^2	3/3	N/A	3/3	N/A
	10^1	3/3	N/A	3/3	N/A
	10^0	3/3	N/A	3/3	N/A
	Overall	15/15 (100%)	N/A	15/15 (100%)	N/A
Dry dog food	10^4	3/3	3/3	3/3	3/3
	10^3	3/3	3/3	3/3	3/3
	10^2	3/3	3/3	3/3	3/3
	10^1	3/3	3/3	3/3	3/3
	10^0	3/3	3/3	2/3	3/3
	Overall	15/15 (100%)	15/15 (100%)	14/15 (93%)	15/15 (100%)
Pig ear treats	10^4	3/3	3/3	2/2[c]	2/2[c]
	10^3	3/3	3/3	2/2	2/2
	10^2	3/3	3/3	2/2	2/2
	10^1	3/3	3/3	2/2	2/2
	10^0	3/3	3/3	1/2	2/2
	Overall	15/15 (100%)	15/15 (100%)	9/10 (90%)	10/10 (100%)

[a]Concentration units are CFU/mL for TSB and CFU/25 g for dry dog food and pig ear treats.
[b]Microplate IC includes a 3-h pre-enrichment step.
[c]Data from the third trial of pig ear treats was not used because the negative control tested positive for *Salmonella*.

Compared to the results of selective plating, a slightly lower detection rate of 93% (14/15) was observed for *S.* Infantis in dry dog food samples when microplate IC was combined with LAMP-BART (Table 4). Microplate IC with LAMP-BART showed detection of *S.* Infantis in 100% of samples at levels down to 10^1 CFU/25 g; however, one of the three samples tested at

10^0 CFU/25 g could not be detected. Consistent detection at 10^0 CFU/25g was anticipated to be difficult due to the combination of a low bacterial concentration with the small amount of sample tested. Along these lines, microplate IC combined with LAMP-BART showed a detection rate of 90% (9/10) for *Salmonella* in pig ear treats. Similar to the results found with dog food, microplate IC combined with LAMP-BART showed 100% detection in *Salmonella* at levels at low as 10^1 CFU/25 g, but one of the samples tested at 10^0 CFU/25 g could not be detected. Although the pig ear treats were tested in a series of three trials, data from the third trial could not be used due to the negative control testing positive for *Salmonella*.

The overall rates reported for detection of *S.* Infantis in food samples using microplate IC combined with LAMP-BART (90-93%) were greater than the rate reported by Fakruddin et al. (2017) for detection of *S.* Typhi in minced beef samples (62.7%) using microplate IC combined with PCR. Fakruddin et al. (2017) tested bacterial concentrations of 10^1 CFU/25 to 10^5 CFU/25 and reported detection of only 20% (3/15) of samples at the lowest concentration. In contrast, the current study reported the ability to detect *S.* Infantis at levels as low as 10^0 CFU/25 with rates of 50-66%. The greater detection rates reported in the current study may be due, in part, to the enhanced specificity and sensitivity of LAMP-BART as opposed to PCR. Additionally, the current study utilized a 3-h pre-enrichment period, a 2-h microplate incubation, and did not discard the sample from the microplate prior to testing as was done by Fadrukkin et al. (2017).

The use of a 24-h enrichment period prior to LAMP-BART enabled *Salmonella* detection in 100% of the dog food samples (15/15) and pig ear treats (10/10) tested. These results are consistent with those reported by Yang et al. (2016), who found that after a 24-h period, LAMP-BART positively detected 1-3 CFU/ 25 g in dry dog food.

5. Recommendations for Future Studies

This study was unique as it was the first to examine the use of microplate IC with LAMP-BART and selective plating at low concentrations of 10^0-10^4 CFU/25g. Results showed that when combining a pre-enrichment period with microplate IC, 100% of samples were recovered on XLD. This is helpful to the food industry as selective media and microplate IC are relatively quick and inexpensive methods that can provide visible results within 24 h. When microplate IC is combined with DNA based confirmation testing like LAMP-BART, PCR, or another method a company may already have, then results for contamination can be obtained within one working day. Extending the pre-enrichment period to 4 h may help to increase the sensitivity of the assay. This study was exclusive to *Salmonella* Infantis in dry dog food products so future research can explore other food types, such as raw pet foods. Using the microplate IC method + LAMP-BART and microplate IC + selective plating with different serotypes or different species of bacteria can also be explored. While *Salmonella* is the leading bacterial cause of foodborne illness in the United States, other bacterial species common in food contamination and dangerous to human health, like *Listeria monocytogenes,* can be tested with the methods used in this study.

6. Summary and Conclusions

Overall, the results of this study suggest that microplate IC combined with LAMP-BART or selective plating could be used to shorten detection time for *Salmonella* in food samples. Microplate IC followed by selective plating on XLD enabled consistent detection of *S.* Infantis in all dog food and pet treat samples tested, including at levels of 10^0 CFU/25 g. This reduced the time to detection to 27 h, compared to 48 h using traditional enrichment combined with selective plating. Microplate IC coupled with LAMP-BART enabled consistent detection of *S.* Infantis in dog food and pet treat samples down to levels of 10^1 CFU/25 g. These results indicate that

microplate IC combined with LAMP-BART can potentially be used to detect *Salmonella* at low levels within one working day (8 h), as opposed to 2 days using a 24-h enrichment combined with LAMP-BART. Extending the pre-enrichment time to 4 h could potentially increase the sensitivity of this method while still allowing for detection within one working day. The optimized conditions from this study can be used to test other isolates of the *Salmonella* spp. in dog food and treat products or other food substrates.

7. References Cited

Andrews WH, Wang H, Jacobson A, Hammack T. 2016. Bacteriological Analytical Manual, Chapter 5: Salmonella. Available from: https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070149.htm. Accessed 2017 February 4.

APPA. 2017. Pet Industry Market Size & Ownership Statistics. Available from: http://www.americanpetproducts.org/press_industrytrends.asp. Accessed 2017 May 9.

Arbault P, Desroche N, & Larose D. 2014. Methods for immunocapture and concentration of bacteria in a sample. EP Patent 14710330.

Arbault, P, Larose D, Nexidia JG. 2014. Microplate Immunocapture (MICROPLATE IC): A New Solution for the Isolation/Concentration of Escherichia coli O157:H7 from Food Samples. IAFP 2014. Indianapolis, IN. 3-6 August 2014.

Aryal S. 2015. Urease Test-Principle, Media, Procedure, and Result. Available from: http://www.microbiologyinfo.com/urease-test-principle-media-procedure-and-result/. Accessed 2017 March 14.

Bugarel M, Tudor A, Loneragan GH, Nightingale KK. 2017. Molecular Detection Assay of Five *Salmonella* Serotypes of public interest: Typhimurium, Enteritidis, Newport, Heidelberg,

and Hadar. Journal of Microbiological Methods 134:14-20.

Centers for Disease Control and Prevention (CDC). 2013. Pet Foods and Treats-Tips for Keeping People and Pets Healthy and Safe from *Salmonella*. Available from: https://www.cdc.gov/features/salmonelladrypetfood/. Accessed 2017 February 3.

Centers for Disease Control and Prevention (CDC). 2018. Pet Food Safety. Available from: https://www.cdc.gov/features/salmonelladrypetfood/. Accessed 2018 August 17.

Centers for Disease Control and Prevention (CDC). 2019. Salmonella. Available from: https://www.cdc.gov/salmonella/index.html. Accessed 2019 January 20.

Davies RH, Wales AD. 2013. *Salmonella* Contamination of Cereal Ingredients for Animal Feeds. Veterinary Microbiology 166(3-4): 543-49.

Diamond. 2017. Maintenance. Available from: http://www.diamondpet.com/our-brands/diamond/maintenance/. Accessed 2017 February 4.

Fakruddin M, Hossain MN, Ahmed MM. 2017. Evaluation of microplate immunocapture, method for detection of *Vibrio cholerae*, *Salmonella* Typhi and *Shigella flexneri* from food. BMC Microbiology 17(189): 1-9.

Food and Drug Administration (FDA). 2012. Bad Bug Book, Foodborne Pathogenic Microorganisms and Natural Toxins. Second Edition. [*Salmonella* species, pp. 9-13].

Food and Drug Administration (FDA). 2015. Hazard Analysis and Risk-Based Preventative Controls for Human Food: Draft Guidance for Industry. Available from: https://www.fda.gov/downloads/Food/GuidanceRegulation/FSMA/UCM517405.pdf. Accessed 2019 January 22.

Food and Drug Administration (FDA). 2017. Think Food Safety and be *Salmonella* Safe. Available from: https://www.fda.gov/ForConsumers/ConsumerUpdates/ucm278271.htm.

Accessed 2019 January 20.

Food and Drug Administration (FDA). 2018a. Foodborne Illnesses: What You Need to Know. Available from: https://www.fda.gov/Food/ResourcesForYou/Consumers/ucm103263.htm. Accessed 2018 August 17.

Food and Drug Administration (FDA). 2018b. Pet Food. Available from: https://www.fda.gov/animalveterinary/products/animalfoodfeeds/petfood/ucm2006475. Accessed 2018 August 1.

Food and Drug Administration (FDA). 2018c. Recalls and Withdrawals. Available from: https://www.fda.gov/AnimalVeterinary/SafetyHealth/RecallsWithdrawals/default.htm. Accessed 2018 August 12.

Gandelman OA, Church VL, Moore CA, Kiddle G, Carne CA, Parmar S, Jalal H, Tisi LC, Murray JAH. 2010. Novel Bioluminescent Quantitative Detection of Nucleic Acid Amplification in Real-Time. PLos ONE 5(11): e14155.

Gruzdev N, Pinto R, Sela S. 2011. Effect of Desiccation on Tolerance of *Salmonella Enterica* to Multiple Stresses. Applied and Environmental Microbiology 77(5): 1667-73.

Hara-Kudo Y, Kumagai S, Masuda T, Goto K, Ohtsuka K, Masaki H, Tanaka H, Tanno K, Miyahara M, Konuma H. 2001. Detection of *Salmonella Enteritidis* in Shell and Liquid Eggs using Enrichment and Plating. International Journal of Food Microbiology 64:395-99.

Huang TM, Lin TL, Wu CC. 2009. Antimicrobial Susceptibility and Resistance of Chicken *Escherichia coli*, *Salmonella* spp., and *Pasteurella multocida* Isolates. Avian Disease 53(1): 89-93.

Imanishi M, Rotstein DS., Reimschuessel R, Schwensohn CA, Woody Jr. DH, Davis SW, Hunt AD, Arends KD, Achen M, Cui J, Zhang Y, Denny LF, Phan QN, Joseph LA, Tuite CC, Tataryn JR, Behravesh CB. 2014. Outbreak of *Salmonella* enterica serotype Infantis infection in humans linked to dry dog food in the United States and Canada, 2012. Journal of the American Veterinary Medical Association. 244(5): 545-53.

Kumar Y, Bansal S, Jaiswal P. 2012. Loop-mediated isothermal amplification (LAMP): A rapid and sensitive tool for quality assessment of meat products. Comprehensive Reviews in Food Science and Food Safety. 16(6): 1359-78.

Lambertini E, Mishra A, Guo M, Cao H, Buchanan RL, Pradhan AK. 2016. Modeling the Long-term Kinetics of *Salmonella* Survival on Dry Pet Food. Food Microbiology 58: 1-6.

Lawley R. 2013. *Salmonella*. Available from: http://www.foodsafetywatch.org/factsheets/salmonella. Accessed 2017 March 13.

Li X, Bethune LA, Jia Y, Lovell RA, Proescholdt TA, Benz SA, Schell TC, Kaplan G, McChesney DG. 2012. Surveillance of *Salmonella* Prevalence in Animal Feeds and Characterization of the *Salmonella* Isolates by Serotyping and Antimicrobial Susceptibility. Foodborne Pathogens and Disease 9(8):692-8.

Margot H, Stephan R, Guarino S, Jagadeesan B, Chilton D, O'Mahoney E, Iversen C. 2013. Inclusivity, Exclusivity, and Limit of Detection of Commercially Available Real-time PCR Assays for Detection of *Salmonella.* International Journal of Food Microbiology 165(3): 221-26.

Nemser SM, Doran T, Grabenstein M, McConnell T, McGrath T, Pamboukian R, Smith AC, Achen M, Danzeisen G, Kim S, Liu Y, Robeson S, Rosario G, McWilliams Wilson K, Reimschuessel R. 2014. Investigation of *Listeria*, *Salmonella*, and Toxigenic *Escherichia*

Coli in Various Pet Foods. Foodborne Pathogens and Disease 11(9): 706-09.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. 2000. Loop-mediated Isothermal Amplification of DNA. Nucleic Acids Research 28: e63.

Rogers SA, Calicchia M, Hellberg RS. 2018. Concentration of *Listeria monocytogenes* in skim milk and soft cheese through microplate immunocapture. Journal of Microbiological Methods 153: 54-59.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. 2011. Foodborne Illness Acquired in the United States-Major Pathogens. Emerging Infectious Diseases 17(1): 7-15.

USDA. 2012. Introduction to the Microbiology of Food Processing. Chapter: Foodborne Pathogens. Available from: http://www.foodsafetywatch.org/factsheets/salmonella/. Accessed 2017 March 13.

USDA. 2016. *Salmonella* and Salmonellosis. Available from: https://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/foodborne-illness-and-disease/salmonella/sa. Accessed 2017 February 2.

Wang L, Shi L, Alam MJ, Geng Y, Li L. 2008. Specific and Rapid Detection of Foodborne *Salmonella* by Loop-mediated Isothermal Amplification Method. Food Research International 41(1): 69-74.

Wang F, Jiang L, Ge B. 2012. Loop-mediated isothermal amplification assays for detecting shiga-toxin producing Escherichia coli in ground beef and human stools. Journal of Clinical Microbiology 50(1): 91-7**.**

Yang Q, Wang F, Prinyawiwatkul BG. 2014. Robustness of *Salmonella* Loop-mediated

Isothermal Amplification Assays for Food Application. Journal of App Microbiology 116(1): 81-8.

Yang Q, Domesle KJ, Wang F, Ge B. 2016. Rapid Detection of *Salmonella* in Food and Feed by Coupling Loop-mediated Isothermal Amplification with Bioluminescent Assay in Real-time. BMC Microbiology 16(112): 1-10.

Zicker, S. 2008. Evaluating Pet Foods: How Confident are you When You Recommend a Commercial Pet Food? Topics in Companion Animal Medicine 23(3): 121-26.