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Molecular detection assay of five Salmonella serotypes of public interest: Typhimurium, Enteritidis, Newport, Heidelberg, and Hadar

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ABSTRACT

Foodborne illnesses due to Salmonella represent an important public-health concern worldwide. In the United States, a majority of Salmonella infections are associated with a small number of serotypes. Furthermore, some serotypes that are overrepresented among human disease are also associated with multi-drug resistance phenotypes. Rapid detection of serotypes of public-health concern might help reduce the burden of salmonellosis cases and limit exposure to multi-drug resistant Salmonella.

We developed a two-step real-time PCR-based rapid method for the identification and detection of five Salmonella serotypes that are either overrepresented in human disease or frequently associated with multi-drug resistance, including serotypes Enteritidis, Typhimurium, Newport, Hadar, and Heidelberg. Two sets of four markers were developed to detect and differentiate the five serotypes. The first set of markers was developed as a screening step to detect the five serotypes; whereas, the second set was used to further distinguish serotypes Heidelberg, Newport and Hadar. The utilization of these markers on a two-step investigation strategy provides a diagnostic specificity of 97% for the detection of Typhimurium, Enteritidis, Heidelberg, Infantis, Newport and Hadar. The diagnostic sensitivity of the detection makers is >96%. The availability of this two-step rapid method will facilitate specific detection of Salmonella serotypes that contribute to a significant proportion of human disease and carry antimicrobial resistance.

1. Introduction

Foodborne diseases in general, and salmonellosis in particular, result in an important economic burden worldwide due to employee absenteeism, treatment, hospitalization, and mortality, with *Salmonella* reported to cause the largest burden on public health in the United States (Batz et al., 2012; Scallan et al., 2011). Because of its zoonotic nature, *Salmonella* also results in substantial economical and animal losses for farmers (Scallan et al., 2011; Scharff, 2012). Further, the ability of *Salmonella* to proliferate in a large variety of conditions makes it able to contaminate a wide variety of food products contributing to its importance in public health.

The United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) dictates that *Salmonella* is an adulterant in ready-to-eat meat and poultry products and therefore has established a required testing policy regarding this pathogen (FDA, 2011a, 2011b, 2014a). Meat and poultry are subject to a zero tolerance policy, in regard to visible fecal contamination on carcass surfaces during the slaughtering process (USDA:FSIS, 1998). *Salmonella* is not normally considered an adulterant, but rather, its presence in various raw products is used as a performance standard in order to evaluate the effectiveness of sanitary slaughter procedures (USDA:FSIS, 2011). Furthermore, as part of the national school lunch program (NSLP), the agricultural marketing service (AMS) of the USDA requires ground beef suppliers to adhere to strict tolerances for *Salmonella*, that is used as measurement of food safety. Regarding meat products supposed to enter the NSLP, *Salmonella* is considered as an adulterant in raw ground beef, and trim (Ollinger et al., 2014). Performance of raw chicken suppliers for the NSLP has also improved regarding *Salmonella* contamination (Ollinger et al., 2015).

Salmonella is a highly diverse genus, which is divided into two species: *S. enterica* and *S. bongori*. *S. enterica* is the most diverse, frequently encountered, and is subdivided into 6 subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI), with the majority of *Salmonella* serotypes belonging to *S. enterica* subsp. *enterica* (>1500) (Grimont and Weill, 2007). Because of its status of adulterant in ready-to-eat meat and poultry products, multiple molecular assays have been developed to detect the presence of *Salmonella* genus strains on food samples. Such molecular assays are often based on the detection of conserved genes, such as *invA* or *ttrC* (Bugarel et al., 2011; Chiu and Ou, 1996; Soto et al., 2006; Wang et al., 1997). However, despite this large diversity, only a small number of serotypes are

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overrepresented in human clinical cases. The national surveillance annual summary, published by the Centers for Disease Control and Prevention (CDC), identified the three serotypes: Enteritidis, Typhimurium and Newport, to be the most frequently encountered and to cause the greatest disease burden on consumers in the United States between 1999 and 2009. Together, these 3 serotypes represented 41.8% and 44% of the clinical isolates reported by the CDC in 2009 (CDC, 2011) and by the CDC-FoodNet in 2013 (CDC-FoodNet, 2013), respectively. Furthermore, the CDC reported that the four most threatening serotypes of *Salmonella enterica* to public health are Typhimurium, Newport, Hadar and Heidelberg, because of their association with multidrug resistance (MDR) (CDC, 2011). The contamination of food products with MDR pathogenic bacterial strains represents an important public health concern. Antibiotic resistant foodborne pathogens can be associated with an increased risk of hospitalization for infected people. Furthermore, a large proportion of resistance mechanisms are encoded on genetic mobile elements, which can be transmitted horizontally to other bacteria potentially present in the intestinal tract. More specifically recent reports show that a high proportion of *Salmonella* strains belonging to Heidelberg, Newport and Typhimurium serotypes from retail meat and poultry products are multi-drug resistant (CDC, 2014b; FDA, 2014a, 2014b).

The CDC considers MDR *Salmonella* a serious threat that requires rapid and efficient actions to avoid aggravation of the situation (CDC, 2014a, 2014b). In response, a petition declared specific MDR strains of *Salmonella* to be considered adulterants in raw ground meat and poultry products, including *S.* Heidelberg, *S.* Hadar, *S.* Newport and *S.* Typhimurium (Citizen-Petition, 2011; DeLauro and Slaughter, 2014). Raw meat products are highly perishable and a decision on how to handle the product has to be made based on a rapid method to detect the presence or absence of molecular markers by screening a sample directly after selective enrichment. Assays have been already developed for the overall detection of *Salmonella* genus. However, there is no existing rapid method to screen sample enrichments to detect and identify these *Salmonella* serotypes. Between March 2013 and July 2014, a large outbreak involving 634 persons from 29 different states and Puerto Rico was reported. This major outbreak involving seven strains of MDR *S.* Heidelberg was linked to the consumption of poultry products (CDC, 2014a, 2014b). This outbreak highlighted the need for specific and rapid methods to detect MDR *Salmonella* serotypes associated with a significant public health burden to facilitate outbreak detection and microbial source tracking to shortly identify the food vehicle responsible for disease and mitigate the dissemination of such threatening pathogens.

Multiple molecular assays to determine the serotype of *Salmonella* isolates have already been developed (Fitzgerald et al., 2007; Franklin et al., 2011; Herrera-Leon et al., 2007; Luk et al., 1993; McQuiston et al., 2004; Mortimer et al., 2004; Yoshida et al., 2016). However, these assays are based on the identification of the somatic and flagellar antigens, which determine antigenic formula thus following the Kauffmann-White-Le Minor scheme (KWM). The KWM scheme summarizes *Salmonella* nomenclature and all the *Salmonella* antigenic formulae encountered to date, encompassing >2600 serotypes (Grimont and Weill, 2007).

Currently, 46 somatic and 114 flagellar antigens have been identified in *Salmonella* genus. Genes involved in the expression of somatic antigens are grouped into a large regulon called the *rfb* cluster. In particular, this cluster contains *wzx* and *wzy* genes encoding for O-antigen flippase and polymerase, respectively. These genes are frequently targeted for the design of serogrouping markers in molecular assays mimicking the KWM scheme (Fitzgerald et al., 2007; Herrera-Leon et al., 2007; Luk et al., 1993). Genes encoding for the flagellin structural proteins are *fliC* (phase 1 flagellin) and *fljB* (phase 2 flagellin). These genes are also frequent targets for the identification of the flagellar variants in molecular serotyping protocols following the KWM scheme (Franklin et al., 2011; McQuiston et al., 2004; Mortimer et al., 2004). Major drawbacks of this kind of molecular serotype determination approaches are linked to their complexity as they are following the KWM workflow. Furthermore, given that they require the utilization of several markers for the identification of a serotype they have to be used on isolated strains and not directly on complex matrices, such as food products or human stools.

In this study, we focused on the development of single markers to detect and identify five important *Salmonella* serotypes chosen based on their association with human disease, their potential role in spreading antimicrobial resistance, and their potential future involvement in regulation.

We developed a two-step rapid detection and identification method for *Salmonella* serotypes: Typhimurium, Heidelberg, Newport and Hadar as a tool to address the petition in the event it spurs regulatory action. In addition, we included *S.* Enteritidis in our method, although primarily characterized as pan-susceptible to tested antimicrobials, this serotype alone accounts for 17.5% of human cases and it is frequently associated with large multi-state outbreaks of foodborne illness attributed to poultry products (CDC, 2011). The present study focusses on the evaluation of these newly developed markers as direct detection assay of these five important *Salmonella* serotypes on complex matrix such as ground beef samples.

2. Material and methods

2.1. Strain collection

A total of 447 strains belonging to 149 serotypes were investigated in this study. The characteristics of these strains are presented on Supplemental Table 1. Of these, 205 were from the Food Microbe Tracker strain collection at Cornell University (Ithaca, New York), 82 were supplied by the French Agency for Food, Environmental and Occupational Health & Safety (Anses, at Maisons-Alfort, France) and had been isolated from food products in France, and the remaining were available to us in the isolate collection of the International Center for Food Industry Excellence (ICFIE) laboratory in the department of Animal and Food Sciences at Texas Tech University (Lubbock, Texas).

All isolates used in this study had been previously serotyped using the conventional approach in France (Anses, Maisons-Alfort) or at Cornell University (Ithaca, NY) or using a hybrid method combining the phenotypic identification of somatic antigens and the molecular identification of flagellar antigens (USDA, Nebraska).

The investigated collection was composed of an inclusivity panel grouping together a minimum of 25 strains belonging to the five serotypes of interest: Enteritidis (n = 26), Typhimurium (n = 25), Newport (n = 30), Hadar (n = 25), and Heidelberg (n = 27), and an exclusivity panel containing 121 serotypes from *S. enterica* subsp. *enterica* together with few strains of the 5 other *Salmonella* subspecies. The exclusivity panel counted up to 5 strains for each of the 30 most prevalent serotypes in human salmonellosis according to the CDC report (CDC, 2011). Furthermore, we also included outside genus isolates from the *Enterobacteriaceae* family as well as similar organisms to *Salmonella enterica*. A total of 13 different non-*Salmonella* genera were included on this study.

2.2. DNA extraction methods

DNA extractions used in this study were either provided by the French Food Safety Agency or were performed either by the boiling extraction protocol or following the manufacturer's instructions of the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO). Genomic DNA extractions were quantified using the Nanodrop 2000c Spectrophotometer (ThermoScientific, Waltham, MA).