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Rapid real-time loop-mediated isothermal amplification combined with coated activated carbon for detection of low numbers of *Salmonella enterica* from lettuce without enrichment

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A real-time loop amplified (Rti-LAMP) DNA assay system was developed for the rapid detection of low numbers of *Salmonella enterica ser.* Enteritidis (*S. enterica*) on the leaves of romaine lettuce without enrichment. The assay involved seeding 50 g portions of leaves with various numbers of *S. enterica* CFU. The lowest level of DNA consistently detected by the Rti-LAMP assay was that from 25 CFU per 50 g (0.5 CFU/g) of lettuce equivalent to the DNA from 6 CFU per Rti-LAMP reaction. A standard curve was generated by plotting the Tp values (min) against the log of 6, 25, 60 and 250 CFU of *S. enterica* seeded onto 50 g of lettuce. The entire assay could be completed in 3.5 h.7

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1. Introduction

The contamination of vegetables such as lettuce is an important cause of infection by enteropathogenic bacteria, causing numerous outbreaks and costly recalls during the past decade. The presence of *Salmonella* on raw vegetables is presently an important issue of food safety. Currently, the majority of quality control systems available for the surveillance of vegetables in the food industry use an initial pre-enrichment followed by a detection system with selective media, and identification by the polymerase chain reaction (PCR) analysis or immunological systems (Chen & Jiang, 2014; Torlak, Akan, & Inal, 2012). Such methodology precludes assessing the presence of pathogens before the product is shipped.

Rapid detection methodologies for infectious organisms such as *Salmonella* have been developed based on the polymerase chain reaction (PCR) (Bohaychuk, Gensler, McFall, King, & Renter, 2007; Rychlik et al., 1999), but can be limited in sensitivity by DNA amplification inhibitors as well as the loss of target cells during their extraction from foods (Rossen, Norskov, Holmstrom, & Rasmussen, 1992; Wilson, 1997). In plant tissues, various compounds including polysaccharides, phenolic compounds and chlorophyll can inhibit DNA amplification (Lee & Levin, 2011; Wilson, 1997). Attachment of bacteria to complex biological surfaces, such as plant tissues involves numerous mechanisms. Researchers have reported that surfactants remove cells attached to hydrophobic surfaces such as plant leaves (Hassan & Frank, 2003; Paul & Jeffrey, 1985).

Activated carbon exhibits a high degree of porosity and an extended interparticulate surface area which results in a high level of adsorption capacity which can be used to efficiently remove DNA amplification inhibitors. The adsorption capacity is strongly influenced by the chemical structure of the carbon surface (Bansal & Goyal, 2005). However, because of the high level of adsorption capacity, the surface of the activated carbon must be adequately blocked so as not to bind targeted bacterial cells. Previous studies (Lee & Levin, 2011; Luan & Levin, 2008) have shown that the recovery of *Escherichia coli* O157:H7 was significantly enhanced by the use of bentonite coated activated carbon (BCAC).

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Unlike the PCR, loop-mediated amplification (LAMP) utilizes a DNA polymerase isothermally at 60–65 °C, and a set of six primers that recognize a total of six distinct sequences on the target DNA (Notomi et al., 2000). An inner primer containing sequences of the sense & antisense strands of target DNA initiates LAMP. The resulting strand displacement DNA synthesis primed by an outer primer releases a single-strand of DNA. This serves as a template for DNA synthesis primed by the second inner & outer primers that hybridize to the other end of the target, which produces a stem-loop DNA structure. In subsequent LAMP cycling, one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA with a stem twice as long. The cycling reaction continues with accumulation of ~10^9 copies of target DNA in less than 60 min. The final amplification products are mixtures of many different sizes of loop DNA, with several inverted repeats of the target sequence and cauliflower-like structures with multiple loops. The identity of the DNA polymerase is a critical factor for efficient amplification. *Bst* polymerase has been found best. *Bst* polymerase (derived from *Bacillus stearothermophilus*) has a helicase-like activity, making it able to unwind DNA strands and facilitating displacement. In addition, it lacks 5′ to 3′ DNase processing activity and will therefore not hydrolyze previously synthesized DNA strands it is displacing, which is an absolutely essential property of the enzyme. Its optimum functional temperature is between 60 and 65 °C and it is denatured at temperatures above 70 °C. These features make *Bst* polymerase useful in LAMP. The method is capable of yielding an unusually large amount of DNA, more than 500 mg/ml. At the completion of amplification a white precipitate of magnesium pyrophosphate forms which can be used to confirm amplification. The yield of synthesized DNA can be quantified by measuring the intensity of fluorescence using ethidium bromide in agarose gels or in a real-time PCR (Rti-PCR) unit or alternatively by measuring the turbidity. The specificity of amplification can be readily distinguished from non-specific amplification by different banding patterns on agarose gels. Quantification of in-put target DNA in Rti-LAMP assays is based on plotting the Tp value on the Y axis and the log of input targets on the X axis. The Tp value is defined as the time in minutes of incubation required for the optical system to just detect newly synthesized DNA and for the amplification plot to traverse the fluorescent baseline. The Tp value in Rti-LAMP replaces the CT value in conventional Rti-PCR.

The purpose of this study was to further develop a rapid and highly sensitive molecular method utilizing a real-time loop amplified (Rti-LAMP) assay for rapid detection of *Salmonella enterica* on Romaine lettuce leaves without enrichment.

2. Materials and methods

2.1. Cultivation of S. enterica

S. enterica serovar Enteritidis strain ATCC BAA-708 (*S. enterica*) was used throughout these studies. All media were obtained from Difco. *S. enterica* was cultured overnight (O/N) in 250 ml flasks containing 50 ml of Tryptic Soy Broth supplemented with 0.5% glucose (TSB^+) at 37 °C with rotary agitation (150 rpm). The next day, a flask containing 50 ml of fresh TSB^+ was inoculated with 1 ml of the O/N culture and similarly incubated to the mid-log phase of growth to an absorbance of ~0.50 (A_{600} of $0.5 = 1.2 \times 10^8$ CFU/ml) in cuvets of 1 cm light path. Bacterial growth was terminated by placing the culture flask in an iced bath. A portion (1.0 ml) of the culture was appropriately diluted with 9.0 ml of chilled saline solution (0.85% NaCl, cat no. S9625, Sigma) and then diluted to the desired CFU/ml and kept on ice prior to use. The correlation between CFU/ml and A_{600} was determined by spread plating 0.1 ml of appropriate dilutions onto duplicate plates of Tryptic Soy Agar supplemented with 0.5% dextrose (TSA^+). After incubation at 37 °C for 16–18 h, the number of colonies was counted. All experiments were replicated at least three times and the resulting means and standard deviations presented.

2.2. Preparation of activated carbon coated with bentonite

Activated carbon (Filtrasorb 200, cat. no. 7440-44-0, Calgon Carbon Corp., Pittsburgh, PA, USA) consisting of 1–2 mm particles was washed several times in bulk with dH_2O in a 1 L beaker until the drained water became clear. Washed activated carbon (16.0 g dry weight) was mixed with bentonite prepared as described below in a 1 L beaker. Four grams of bentonite (Fisher Scientific, cat. no. B-235 71381) was transferred into separate 500 ml blender bottles containing 200 ml of dH_2O and vigorously suspended with an Osterizer blender at high speed for 1 min. The suspensions were then transferred to 250 ml centrifuge bottles and centrifuged at 700 rpm (80 g) for 1 min at room temperature to sediment large particles. The supernatant (~120 ml) which contained about 1.6 g of bentonite was carefully transferred to the beaker containing washed activated carbon. The beaker was placed onto a rotary shaker (150 rpm) at 37 °C for 3 h and then transferred to a 55 °C incubator until the activated carbon coated with bentonite (BCAC) was dry.

2.3. Preparation of lettuce samples

Romaine lettuce (*Lactuca sativa* L. var. longifolia) was purchased from a local retail source. Outer leaves were randomly selected unless otherwise indicated. Leaves were cut into several pieces using a sterile scissors.

2.4. Determination of the percent recovery of the total bacterial CFU in lettuce rinsates using SDS solution followed by different amounts of BCAC

The percent of recovered CFU in lettuce rinsates for 50 g samples of lettuce following treatment with different amounts of BCAC was determined by first placing 150 g of lettuce leaves into a sterile plastic drum (8 L). Three hundred ml of 0.025% sodium dodecyl sulfate (SDS) solution were added to the sample to remove resident bacterial cells from the lettuce. The sample was swirled vigorously 100 times (~90 s) and the rinsate was passed through a 60 ml syringe barrels containing glass wool (0.5 g) to remove large debris. SDS solution (0.025%, 150 ml) was added to rinse the sample again and the rinsate passed through the same syringe barrel. The combined rinsate was centrifuged at 13,000 g for 15 min and the pellet was suspended in 75 ml of 0.01 M acetate buffered saline (0.85% NaCl, acetic acid: sodium acetate, pH 5.0; ABS). The optical density at 600 nm was measured in a spectrophotometer using cuvets of 1 cm path length, with ABS solution as a blank.

Dry BCAC (2.0, 3.0 and 4.0 g) was transferred to a sterile 250 ml beaker and washed 3 times with 30 ml of ABS. The 75 ml rinsate was divided into three 25 ml portions (each representing 50 g of lettuce) which were mixed with one of the three varying quantities of washed BCAC preparations in 250 ml beakers and then subjected to rotary agitation (150 rpm) for 15 min. Each sample was transferred to a 60 ml syringe barrel containing 0.3 g of glass wool and then eluted with ABS saline until 25 ml was collected in a sterile beaker. The optical density at 600 nm of each sample was measured. In addition, each sample was serially diluted with 0.85% NaCl and 0.1 ml plated in duplicate on TSA^+ plates for total CFU recovery following incubation at 20 °C for 3 days, and colonies

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