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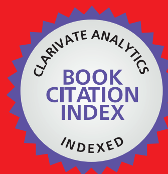

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Chapter 7

PCR Assay for Detection of *Staphylococcus aureus* in Fresh Lettuce (*Lactuca sativa*)

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Additional information is available at the end of the chapter

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Abstract

The growth in food demand and production growth of vegetables have led to the development of intensive production systems with the aim of having regular access to enough high-quality food. The aim is to determine the incidence of *Staphylococcus aureus* in fresh lettuce by PCR in order to enhance the efficiency for detection and identification process. The Baird-Parker method was used for isolating pathogens from 54 lettuce samples. Genomic DNA extraction was performed according the Mericon DNA Bacteria Plus Kit. The detection by PCR was performed using the pair of primers: *coa* gene (5′-ATAGAGCTGATGGTACAGG-3′ and 5′-GCTTCCGATTGTTCGATGC-3′). The phylogenetic tree was constructed by comparing conserved sequences from the adjacent 16S gene, using the F2C 5′-AGAGTTTGATCATGGCTC-3′ and C 5′-ACGGGCGGTGTGTAC-3′ primers. To test the antimicrobial effect, we used the disk diffusion method (Kirby-Bauer) using Mueller-Hinton agar and five antibiotics with different concentrations. The incidence of *S. aureus* was 1.7%. All the isolates were situated in the ATCC 11632 clade in accordance with other reported sequences belonging to this pathogen in the NCBI database. All the isolates seemed to be resistant to penicillin (10U). The molecular techniques used in this study are suitable for the identification of *S. aureus* isolated from lettuce, increasing our capability of detecting this pathogen by improving the process and increasing the efficiency contributing to the safety of this vegetable.

Keywords: *S. aureus*, fresh produce, lettuce, PCR, *coa* gene

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1. Introduction

1.1. Taxonomy

Staphylococcus is a member of the *Micrococcaceae* family and consists of Gram-positive cocci, catalase-positive, that usually presents oxidative and fermentative metabolism of glucose. With measurements around 1 μm in diameter, their cells show a characteristic cluster of irregular arrangement as a result of cell division and some produce carotenoid pigments in yellow or golden colors [1, 2].

They are coagulase variant, non-spore-forming, facultative anaerobes (except *Staphylococcus saccharolyticus* reported to exhibit a faster and abundant development under aerobic conditions). *Staphylococcus* is catalase variant commonly positive (*Staphylococcus aureus* subesp. *anaerobius* and *S. saccharolyticus* are catalases negative), with Gram-variable capsule usually negative, (but always present), immobile, and oxidase variant commonly negative (*Staphylococcus caseolyticus*, *Staphylococcus lentus*, *Staphylococcus sciuri* and *Staphylococcus vitulus* are positive to the modified oxidase reaction). A O/F glucose test determines them as F (fermentative) microorganisms, with optimum growth temperature from 30 to 37°C. There are 44 species sensitive to lysis by lysostaphin, but resistant to lysozyme (*S. arlettae*, *S. aureus* subesp. *anaerobius*, *S. aureus* subesp. *aureus*, *S. auricularis*, *S. capitis* subesp. *capitis*, *S. capitis* subesp. *ureolyticus*, *S. caprae*, *S. carnosus*, *S. caseolyticus*, *S. chromogenes*, *S. cohnii* subesp. *cohnii*, *S. cohnii* subesp. *urealyticumk S. delphini*, *S. epidermidis*, *S. equorum*, *S. felis*, *S. gallinarium*, *S. haemoliticus*, *S. hominis*, *S. hyicus*, *S. hyicus* subesp. *hyicus*, *S. intermedius*, *S. kloosii*, *S. lentus*, *S. lugdunensis*, *S. lutrae*, *S. muscae*, *S. pasteuri*, *S. piscifermentans*, *S. pulvereri*, *S. sacharolyticus*, *S. saprophyticus* subesp. *bovis*, *S. saprophyticus* subesp. *saprophyticus*, *S. schleiferi*, *S. schleiferi* subesp. *coagulans*, *S. schleiferi* subesp. *schleiferi*, *S. sciuri*, *S. sciuri* subesp. *carnaticus*, *S. sciuri* subesp. *rodentium*, *S. sciuri* subesp. *sciuri*, *S. simulans*, *S. vitulus*, *S. warneri*, *S. xylosusk*). *S. aureus* subesp. *aureus*, *S. epidermidis*, *S. haemolyticus*, *S. lugdunesis*, *S. saprophyticus* subesp. *saprophyticus* are species of *Staphylococcus* isolated most frequently associated with human infections [1]).

S. aureus is considered a pathogen with high potential to cause multiple infections in humans and animals; it was discovered by Dr. Alexander Ogston in 1880 [3]. Being the most common pathogen causing nosocomial infections [4, 5], as well as cases of food poisoning [6], it is considered the most virulent bacteria, responsible for a broad spectrum of diseases, ranging from skin and soft tissue infections to serious diseases that threaten life. *S. aureus* belongs to the normal human flora, and 25–50% of the healthy population is colonized with this bacterium, constituting a risk of dissemination, as it can be acquired through contact with other people or through environmental exposure [3].

Pathogenicity of *S. aureus* infections is related to various components of the bacterial surface generally formed by peptidoglycan and teichoic acids, in addition to protein A [7]. The pathogenesis caused by this microorganism occurs when the combination of virulence factors with decreased host defenses occurs [8], and these conditions favor the microorganism having the characteristics of virulence and damage [9]. In addition, the situation is aggravated because

this pathogen has developed resistance to multiple antibiotics, making the development of novel treatments and medication for diseases caused by this bacterium more challenging [10].

1.2. *S. aureus* in food

The presence of pathogens in minimally processed vegetables and their ability to survive and grow has been well documented [11]. *S. aureus* (including methicillin-resistant *S. aureus*, MRSA) is one of the most common pathogens in food and is considered responsible for the most common types of poisoning in meats, salads, milk, and dairy products [12–14]. A wide variety of foods can be a vehicle for *S. aureus* poisoning; besides pollution exposure (usually human) and the use of raw materials and contaminated ingredients (typically but not exclusively of animal origin), ecological conditions must be met in food to favor growth of the microorganism, including temperatures above 20–25°C, even for a few hours.

Therefore, it is considered that *S. aureus* has a remarkable ability to proliferate in various foods; under conducive conditions, the growth rate can lead to enough concentration of enterotoxins to cause severe outbreaks of gastroenteritis. Meat, dairy products, certain vegetables, and cooked foods work as excellent growth medium to support their multiplication. It is important to mention that not all foods are favorable substrate for the development of the microorganism; in fact, some are inhibitory; development patterns of *S. aureus* in some foods are favorable (raw meat, cooked chicken, fresh cheese, raw milk, yolk), unfavorable (raw vegetables, ground beef, raw fruit, dried fruit, nuts), and inhibitory (chocolate, cocoa, mature dairy, processed juice, fermented food) [12].

There are *S. aureus* strains more pathogenic than others: one example is the methicillin-resistant *S. aureus* (MRSA). Methicillin is a semi-synthetic derivative of penicillin introduced in Europe in 1959; one year after its introduction, the first strain of MRSA was detected, and in 1963, the first nosocomial outbreak caused by this microorganism was reported. MRSA has been thoroughly studied at a genetic level, due to its recurrent appearance in later years. In some cases, it has been associated with food consumption, and knowledge about the spread and epidemiology has been used to develop strategies to prevent the distribution of MRSA. Simultaneously, the development of various molecular typing techniques has emerged, aimed at detecting the phenotypes or specific molecular characteristics of each strain in question [15].

1.3. Staphylococcal intoxication

One of the most important foodborne diseases transmitted around the world is Staphylococcal intoxications; of all outbreaks of food poisoning that occur, on average 20% are due to the consumption of food contaminated with enterotoxins produced by bacteria of the genus *Staphylococcus* and mainly for the *S. aureus* species. The intoxication is characterized by nausea, vomiting, abdominal cramps, malaise, headache, and occasionally diarrhea without the presence of fever. Symptoms can appear 30 min after consumption of the aliment, with the most common incubation period going from 2 to 4 h [2].

Commonly, *S. aureus* has been isolated from plants [16]. Microbial contamination in food can occur due to poor storage procedures, and an increase in temperature during shelf life has

been identified as the cause of microorganism proliferation [11]. Infections by Staphylococcal enterotoxins have constituted the leading cause of foodborne disease in the United States, and these enterotoxins are the leading cause of outbreaks caused by contaminated food in the European Union. In June 2000, Japan reported a mass intoxication of more than 10,000 cases caused by this organism present in milk [6, 17]. The Center for Disease Control and Prevention (US-CDC) reports that consumption of contaminated food with *S. aureus* causes 185,060 cases of infection, 1753 hospitalizations, and two deaths annually just in this country [13].

In the Middle East, many types of vegetables are eaten raw in salads or used as garnish appetizers, and in traditional meals, they are perceived as healthy food; however, in other parts of the world, these raw vegetables have been major contributors of foodborne diseases in recent years [18, 19]. In the United States, green leafy vegetables have been identified as part of the 10 riskiest foods regulated by the Food and Drug Administration (FDA), representing almost 40% of foodborne outbreaks according to data obtained from the Center for Disease Control and Prevention (CDC) [20].

The consumption of green leafy vegetables provides numerous health benefits, and there is a direct relationship between consumption of these vegetables and the reduction of chronic diseases such as hypertension, diabetes, atherosclerosis, and cancer [21]. Currently, most fresh-cut products are washed in chlorinated water (50–200 mg L^{-1} of active chlorine) to reduce the levels of microorganisms. Sodium hypochlorite (NaCl) is the most widely used disinfectant in the fresh-cut industry [22]. The lack of thorough cooking in fresh cuisine can result in foodborne diseases if contaminated by pathogens. Despite these foods being ready to eat, it has been reported that their quality is not satisfactory in Vienna, Austria [23], Johannesburg, South Africa [24], Korea [25], and Catalonia, Spain [26]. Reports show that the main pathogens in ready-to-eat foods include *Listeria monocytogenes*, *S. aureus*, *Bacillus cereus*, *Salmonella* spp., and *Escherichia coli* O157:H7, the last two being involved in most outbreaks caused by fresh fruits and vegetables [27, 28] reported in low doses of 10 and 2–2000 cells, respectively [29, 30].

In Mexico, staphylococcal intoxications are responsible for 45% of the outbreaks caused by food poisoning. These data partially reflect the incidence of this disease in the country, considering that it only represents the outbreaks that have been reported or studied; however, it is useful to show that on a national level, staphylococcal poisoning is a major foodborne [2]. The main places where the outbreaks were reported to occur are at parties or social gatherings, schools or daycare centers, restaurants, and hospitals, in this order of importance [31].

In recent years, Mexico has become one of the most dynamic markets for the US horticultural importers, displacing Japan to third place [32]. From January to April 2014, a total of 51,109 tons of lettuce equivalent to 42.289 mdd were exported, the main exporters being: Guanajuato (56.8%), Nuevo Leon (19.9%), Baja California (17%), Sonora (1.9%), other states (4.4%) [33].

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1.4. Identification of *S. aureus*

Various methods have been developed for the isolation and quantitative identification of *S. aureus*; the selection of the method depends on the type of food, as well as the history of incidence of the pathogen.

Processed foods may contain relatively small numbers of debilitated viable cells, whose presence must be demonstrated by appropriate means (BAM, Bacteriological Analytical Manual) [34].

Among the most important methods used globally are those recommended by the Association of Official Analytical Chemistry (AOAC) (975.55-1976, *S. aureus* in foods. Surface plating) and the Food and Drug Administration (BAM, Bacteriological Analytical Manual Chapter 12: *S. aureus*). In Mexico, the microbiological method to determine the account of *S. aureus* present in national or imported foods is established in the Mexican Official Standard NOM-210 appendix B [35], in accordance with the International Standard ISO 6888-1:1999. Microbiology of food and animal feeding stuffs—horizontal method for the enumeration of coagulase-positive staphylococci (*S. aureus* and other species)—Part 1: Technique using Baird-Parker agar medium.

A brief description of the method includes:

1.4.1. Baird-Parker method

1.4.1.1. Preparation of the samples

Take different portions of the food, transfer 25 g or mL into dilution bottles with 225 mL of phosphate buffer or peptone water to prepare a dilution 1:10, and homogenize for 1 or 2 min in blender or peristaltic homogenizer.

1.4.1.2. Analytical procedure

Transfer 0.1 mL of direct sample with a sterile pipette if liquid, or 0.1 mL of the initial suspension (dilution 10^{-1}) in the case of other products, onto plates with Baird-Parker agar with addition of egg yolk emulsion. Do this in duplicates and repeat this procedure for subsequent dilutions 10^{-2}, 10^{-3} if necessary.

Carefully distribute the inoculum on the agar surface as soon as possible, with a sterile glass rod bent at a right angle, using one for each plate and dilution. The plates must be kept with the top upward until the inoculum is fully absorbed by the agar.

Invert and incubate the plates from 44 to 48 h at 36°C and subsequently search for colonies with typical morphology: black in color, circular, bright, convex, flat from 1 to 2 mm in diameter, showing one opaque zone, wet and with a clear halo.

Select the plates having between 15 and 20 typical and atypical colonies for their confirmation. From each sample, select five typical colonies for confirmation or five atypical colonies to

perform Gram staining. In the case of observing positives bacilli, the colony will be taken as negative for *S. aureus*; on the contrary if cocci are observed, the confirmation will continue.

When the plates contain <15 typical colonies, a note citing "estimated value" must be added to the report of results.

1.4.1.3. Confirmation procedure

Coagulase test: Coagulase is a protein produced by various microorganisms that enables the conversion of fibrinogen to fibrin. In the laboratory, it is used to distinguish between different types of *S. aureus* [36]. Dehydrated rabbit plasma is used for the test (this is rehydrated following the manufacturer's instruction).

For the procedure, select and inoculate each typical colony in a tube with 0.5 mL brain heart infusion broth (BHI) and tubes with Trypticase soy agar (TSA). Simultaneously use a positive control (*S. aureus*) and a negative control (*S. epidermidis*). Incubate at 35 + 1°C in a water bath from 20 to 24 h. Keep the bacterial culture in ATS at temperature no more than 27°C for subsequent tests. Add 0.1 mL of the previous bacterial culture to 0.3 mL of rabbit plasma with EDTA (unless the manufacturer indicates other quantities). Incubate at 35°C in a water bath and observe constantly at intervals of 1 h during the first 4–6 h; if there is no clot formation, observe up to 24 h. Consider a positive result when the clot is completely formed and firm when inverting the tube. On the contrary result, auxiliary tests should be performed such as Gram staining of each bacterial culture, seeking Gram-positive cocci grouped in clusters of grapes, a catalase, and fermentation of glucose and mannitol tests.

Important note: For each new batch of reagents, a coagulation test must be performed on rabbit plasma by adding a drop of 5% calcium chloride to 0.5 mL of reconstituted plasma, forming a clot in 10–15 s.

1.4.1.4. Thermonuclease test

S. aureus produces a thermonuclease enzyme (this capacity is not limited to this species). Detection of staphylococcal thermostable deoxyribonuclease (thermonuclease) in food is used as an indirect test to evident the presence of large amounts of *S. aureus* in food and of staphylococcal enterotoxins.

The production of this enzyme is inhibited by anaerobiosis and is stimulated by the presence of oxygen, it requires calcium ions for its enzymatic activity, its optimal pH is 8.6 and is precipitated with ammonium sulfate. Its thermal stability (resistant to temperatures of 130°C for 16.6 min) is the only association with the growth of *S. aureus*.

When interpreting this test, it is essential to consider the existence of enterotoxigenic strains negatives to both tests. Among the negative coagulase strains, some have developed the ability to synthesize enterotoxins. Although *S. aureus* specie is the typical producer of these toxins, other species exhibit the same behavior: *S. intermedius*, *S. hyicus*, *S. warneri*, *S. epidermidis*, among others [37, 38].

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For this test procedure, slides are prepared with 3 mL of toluidine-DNA blue agar. Using a Pasteur pipette makes equally spaced holes in the agar. In a boiling water bath, heat 0.3 mL of bacterial culture in BHI for 15 min. With the use of a Pasteur pipette transfer a drop of bacterial culture to a hole of toluidine blue agar-DNA. Repeat for each strain including the positive and negative controls. Incubate at 35 ± 1°C in a humid chamber from 4 to 24 h. The appearance of a pink halo of at least 1 mm qualifies as a positive test.

See **Table 1** for the characteristics of *S. aureus*, *S. epidermidis*, and micrococci, in order to identifying the specie isolated or identificated.

Characteristic	***S.aureus***	***S.epidermidis***	**Micrococci**
Catalase activity	+	+	+
Coagulase production	+	–	–
Thermonuclease production	+	–	–
Lysostaphin sensitivity	+	+	–
Anaerobic utilization of glucose	+	+	–
Mannitol	+	–	–

[a]+, most (90% or more) strains are positive; –, most (90% or more) strains are negative.

Table 1. Typical characteristics of *S. aureus, S. epidermidis*, and micrococci[a].

1.4.2. Molecular methods

The molecular identification is centered of detection and sequencing a specific bacterial DNA and used to identify and classify taxonomically several groups of microorganisms, including bacteria by amplification of specific target region by PCR. Another strategy is amplified by PCR-specific genes that belong to certain species, based on specific features like virulence factors or antibiotics resistance genes [39]. The development of identification techniques for a clinical rapid diagnosis is necessary. The PCR is a rapid, sensitive, and less time-consuming than the conventional bacteriological identification methods [40] and is extensively used to identify bacteria isolated from different kind of samples, including foods [41], soil [42], and infected human tissue [43].

1.4.3. Identification based on 16S ribosomal RNA gene

The 16S rRNA gene is part of all bacteria and is commonly used for taxonomic purposes because it is a highly conserved region; the rate of protection from change is assumed to result from the importance as a serious constituent of cell function [44]. Into the sequence of 16Sr RNA gene are indicated variable regions; Chakravorty et al. [45] in his study describe nine regions with sufficient diversity that are suitable for taxonomic analysis; their investigation determined that V1 hypervariable region best differentiated among *S. aureus* and coagulase-negative *Staphylococcus* sp. Linked to the PCR technique, the use of the 16S rRNA DNA fragment, amplified by using specific oligonucleotides, has been proposed; this gene is widely

used for the preparation of phylogenetic trees and useful in finding the evolutionary relationships between two or more individuals [40, 46]. For *S. aureus*, the sequence of 16S ribosomal RNA is reported in the NCBI (National Center for Biotechnology Information) with just over 1.4 kb and access to the GenBank (KP728240.1) was reported by Nazari [47].

1.4.4. Coa gene

S. aureus is the only known bacterium that produces coagulase enzyme, which is determined by the *coa* gene. This gene is considered useful and specific for the identification of *S. aureus* and many years ago had been the principal criteria for the separation of *S. aureus* of other *Staphylococcus* that is only ubicuous in this specie [2, 48]. The study of this gene is directly related to the coagulase test. The sequence of *coa* gene is reported in the NCBI (National Center for Biotechnology Information) with just over 2.0 kb and access to the GenBank is AB436964.1 [49].

2. Study case

2.1. Materials and methods

The aim was to determine the incidence of *S. aureus* in fresh lettuce by PCR in order to enhance the efficiency for detection and identification process.

Study zone. Fifty-four lettuce samples were obtained from a company of fresh products located in Sonora, Mexico (**Figure 1**); all the productive process was evaluated, such as cut area, storage, and transportation.

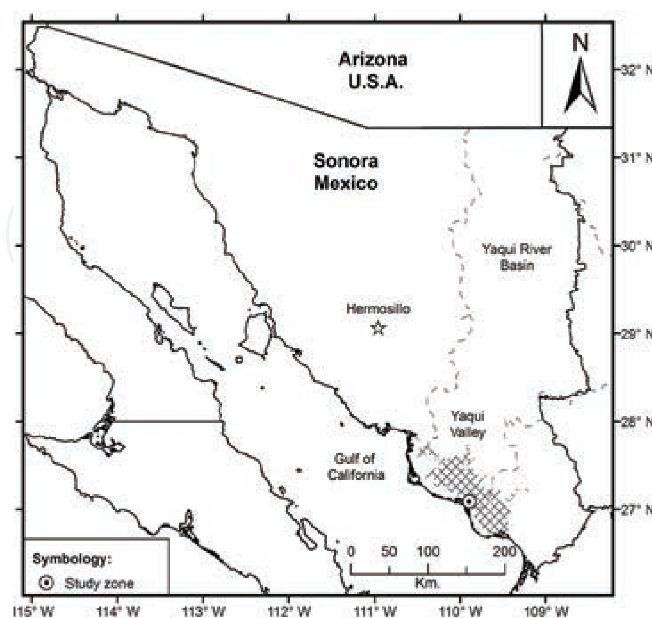

Figure 1. Location of the study area in Sonora, Mexico.

It is important to mention that Mexican vegetable producers strive to be at the top of the market, which involves providing the customers top-quality products all the time. To achieve this goal, they implemented quality control assurance programs as well as partnered with prestigious external certification labs to help they watch every step of the way.

2.2. Transport and handling of sample

Samples were collected as described by Seow et al. [50], briefly, personnel of the laboratory was transferred to the production site in the Yaqui Valley and sampled lettuce from the process, were sampled in original package and immediately placed in sterile resealable bags, and later were transported with iceboxes and stored to 4°C until analysis. Product information such as production date, lote, and "best before" were registered in the database of the study. All the samples were analyzed within 24 h after time of collection, in the meantime, keeping them in their original storage conditions.

2.3. Isolation and identification

The Baird-Parker method was used for isolating pathogens from 54 lettuce samples (**Figure 2**). *S. aureus* ATCC 11632 was used as a positive control; the bacterial strains were cultivated on nutrient agar slant and kept at 4°C. Every 25 days a subculture was carried out to maintain bacterial viability; this process was only repeated two times. A BD Difco™ Tryptic Soy Broth (Soybean-Casein Digest Broth Medium, Ref 211825) was used for the genomic DNA extraction, and the medium was maintained at 30°C with overnight shaking. Genomic DNA extraction was performed according the Mericon DNA Bacteria Plus Kit for Gram-positive bacteria (Qiagen Ref 69534). After extraction procedures, the amount and purity DNA were measured with spectrometer Nanodrop 2000c and the integrity of the DNA on the extracted material in agarose gel electrophoresis was verified.

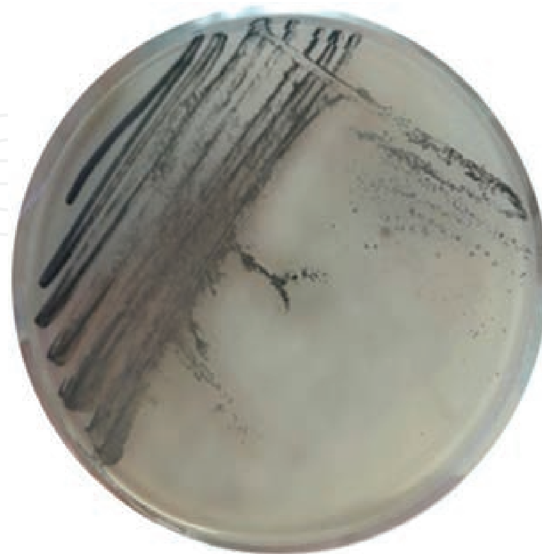

Figure 2. *S. aureus* in Baird-Parker Agar isolated from lettuce.

The detection by PCR was performed using the pair of primers of *coa* gene [48]. In an Eppendorf PCR tube (0.2 mL), 3 mM $MgCl_2$, 0.8 μM oligonucleotides, 1.6 μM dNTP, 1 unit of Taq DNA polymerase, 10 ng/μl ADN, and buffer 1× were added to make a final volume of 25 μl. A fragment of 674 bp was amplified by the primers *coaf* 5′-ATA GAG CTG ATG GTA CAG G-3′ and *coar* 5′-GCT TCC GAT TGT TCG ATG C-3′), and the PCR protocol was performed in a SimpliAmp Thermal Cycler (Applied Biosystem Ref A24812): 94°C for 10 min (1 cycle), 35 cycles of: 94°C for 30 s, 56°C for 1 min and 72°C for 1 min, with a final cycle of 72°C for 5 min.

Additionally, sensitivity test was performed with base in the genomic DNA concentration. The procedure was done according to Shree et al. [51]; briefly, from genomic DNA of the ATCC 11632 were done dilutions (50, 5, 0.5 ng/μl; 50, 5, 0.5 pg/μl and 50, 5, 0.5 fg/μl). PCR and electrophoresis gel were carried out as we described previously for *coa* gene. The assay was done in triplicates.

The phylogenetic tree was constructed by comparing conserved sequences from the adjacent 16S gene, using the F_2C 5′-AGAGTTTGATCATGGCTC-3′ and C 5′-ACGGGCGGTGTGTAC-3′ primers, in order to obtain a fragment of approximately 1600 bp which was bidirectionally sequenced; the mix reaction was as described above and the PCR conditions were as follows: 95°C for 10 min (1 cycle), 32 cycles of: 95°C for 1 min, 60°C for 1 min and 72°C for 2 min, with a final cycle of 72°C for 5 min.

The purification of PCR products was performed according to the Qiaquick PCR Purification Kit (Qiagen, EUA, Ref 28106), and 400 ng was evaporated in a dry bath at 56°C for 12 h for bidirectional sequencing. The sequences of regions were compared with the National Center of Biotechnology Information (NCBI) data (http://www.ncbi.nlm.gov/) using BLAST-N. The output was grouped such that all members exhibited more than 90% similarity; the alignment of the DNA sequence data was analyzed in Mega 6 software for the phylogenetic tree building with bootstrap analysis (1000 repeats).

To test the antimicrobial effect, the disk diffusion method (Kirby-Bauer) was applied, using Mueller-Hinton agar (MCD Ref 7131) and five antibiotics with different concentrations: tetracycline 30 μg (Oxoid Ref CT0054B), trimethoprim-sulfamethoxazole 25 μg (Oxoid Ref CT0052B), clarithromycin 15 μg (Oxoid Ref CT0693B), oxacillin 1 μg (Oxoid Ref CT0159B), and penicillin G 10 U (Oxoid Ref CT0043B). Each assay was performed in triplicate and the diameter of the inhibition zone was calculated (mm) [52].

3. Results

Figure 3a shows the temperature gradient for *coa* gene, where the range of 56–60°C was observed a specific amplification and 56°C was selected temperature to annealing specific primers in target gene. **Figure 3b** shows the sensitivity of the method for target *coa* gene in range of genomic DNA concentrations (50, 5 and 0.5 ng/μl; 50, 5 and 0.5 pg/μl; 50, 5 and 0.5 fg/μl); the method has a sensitivity up to 0.5 pg/μl.

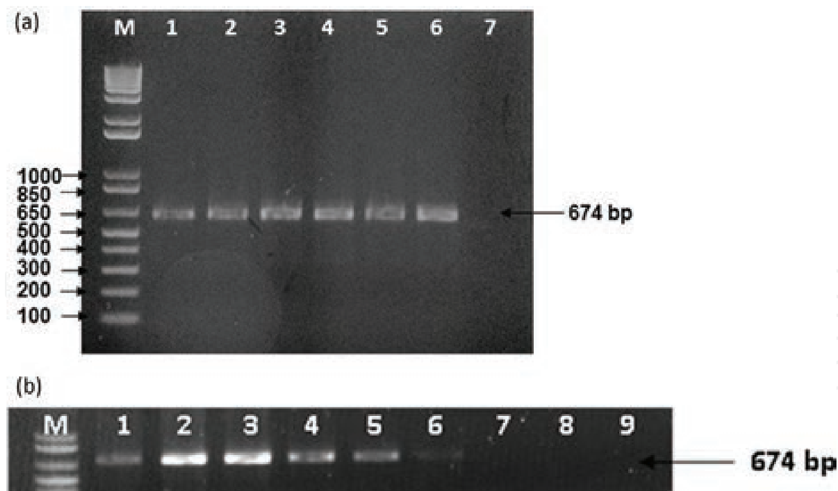

Figure 3. (a) Agarose gel electrophoresis showing temperature gradient for *coa* target gene by PCR with specific primers. M, size marker (1 kb plus DNA Ladder, Invitrogen™); lane 1, 50°C; lane 2, 52°C; lane 3, 54°C; lane 4, 56°C; lane 5, 58°C; lane 6, 60°C and lane 7, negative control (ultrapure water, Invitrogen™). (b) Agarose gel electrophoresis showing the sensitivity of detection for *coa* gene using different DNA concentrations. M, size marker (1 kb plus DNA Ladder, Invitrogen™); lane 1, 50 ng; lane 2, 5 ng; lane 3, 0.5 ng; lane 4, 50 pg; lane 5, 5 pg; lane 6, 0.5 pg; lane 7, 50 fg; lane 8, 5 fg and lane 9, 0.5 fg.

The incidence of *S. aureus* was 1.7% (**Figures 2** and **4**). All the isolates were situated in the ATCC 11632 clade in accordance with other reported sequences belonging to this pathogen in the NCBI database with a bootstrap of 98 (**Figure 5**). Similar reports were given by the GenBank sequences D83357.1, D83355.1, JN315147.1, JN390832.1, JN390831.1, JN315154.1, JN315153.1, JN315151.1, JN315150.1, and JN315149.1 being reported in the same operational taxonomic unit (OTU) with similarities in their morphologic, physiologic, and biochemical characteristics.

All the isolates seemed to be resistant to penicillin G 10 U and were susceptible to oxacillin, tetracycline, clarithromycin, and trimethoprim-sulfamethoxazole (**Figure 6**).

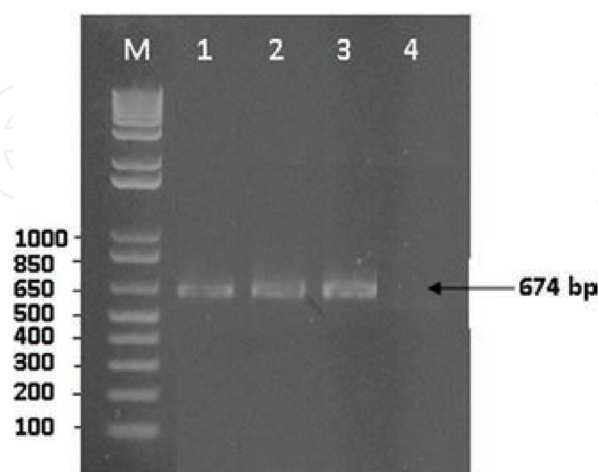

Figure 4. Amplification of target pathogen isolated from lettuce by PCR with specific primers. M, molecular marker (1 kb plus DNA Ladder, Invitrogen™); lane 1, positive control ATCC 11632; line 2, isolated 1; line 3, isolated 2 and line 4, negative control (ultrapure water, Invitrogen™).

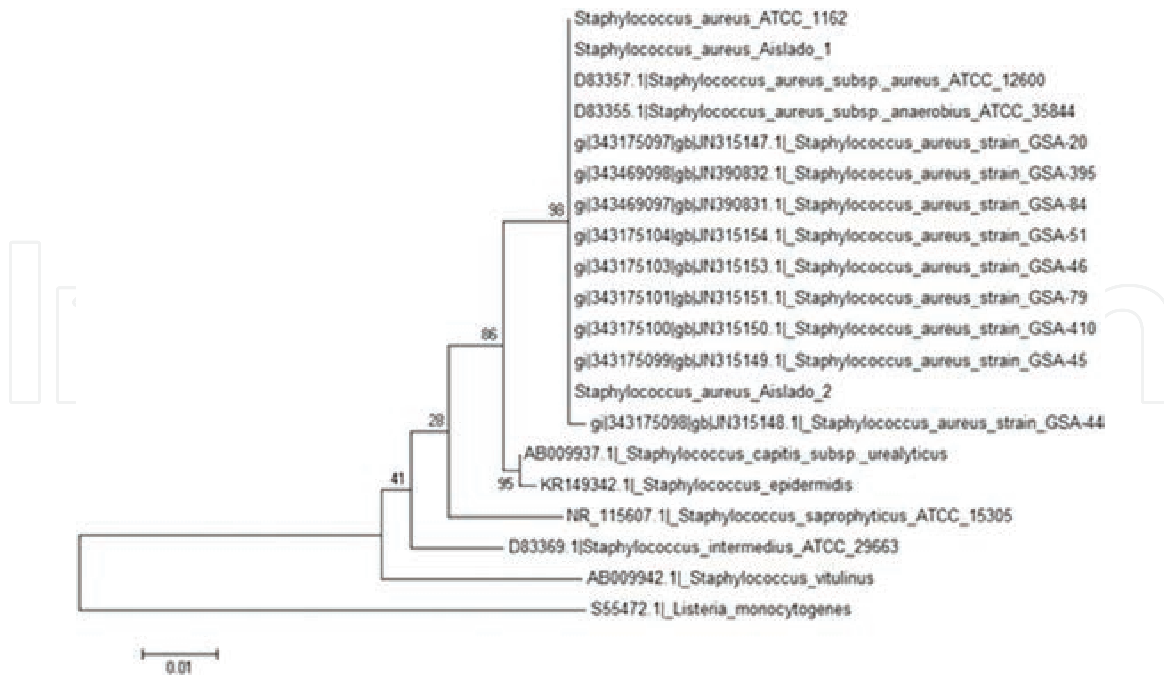

Figure 5. Phylogenetic tree of *S. aureus* maximum verisimilitude constructed from partial sequences of 16S ribosomal DNA of 2 prokaryotic clones and 17 reference sequences obtained from NCBI.

Figure 6. Tests of antibiotic susceptibility; 1: positive control (*Staphylococcus aureus* ATCC11632), 2: isolated 1, 3: isolated 2. (a) Oxacillin 1 μg, (b) trimethoprim-sulfamethoxazole 25 μg, (c) penicillin G 10 U, (d) tetracycline 30 μg, and (e) clarithromycin 15 μg.

4. Discussion

For *coa* gene, the temperature gradient showed that 56°C was the optimal annealing temperature (T_a) for oligonucleotides, showing an adequate specificity for the detection of *S. aureus*. The T_a is defined as the highest temperature where the optimal aligning and amplification occur [53]; this parameter is crucial for the standardization of the method because a low T_a can cause nonspecific amplification, giving undesired PCR products; this is when two or more bands are observed in gel electrophoresis. In this study, the primers features and the correct

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design lead us to obtain a good and specific amplification in a range of 56–60°C. Likewise, a high T_a can cause a low or non-amplification, reducing the possibility to anneal; for this reason, an optimization of priming temperature is necessary [54]. Additionally, annealing was satisfactory at low DNA concentrations (up to 0.5 pg/μl) showing adequate sensitivity. Isolated from lettuce samples were confirmed by amplification of the 674 bp fragment.

For the strategy with the 16S an optimal annealing temperature of 54°C was established for a fragment of approximately 1400 bp; isolates 1 and 2 were aligned in the same clade as the positive control (ATCC 11632) strain. Clinical animal isolates reported at NCBI D83357.1, D83355.1 and isolated from human throats suffering clinical infections JN315147.1, JN390832.1, JN390831.1, JN315154.1, JN315153.1, JN315151.1, JN315150.1, and JN315149.1 show that isolates 1 and 2 are potentially dangerous if the vegetable is not properly sanitized before consuming.

Low incidence of *S. aureus* is directly related to good manufacturing practice of packing companies, mainly because the exposure time of the product in contact with the exterior is very short. Likewise, the product is never in direct contact with the staff due to the use of hairnets, gloves, face masks, aprons ,and boots, as well as all staff washing and disinfecting their hands before entering work and after toileting.

The bacterial counts found in this study were below the health limit of 10^2–10^3 CFU g^{-1} of *S. aureus* in food set by the Codex Alimentarius, stabilizing a good quality of lettuce with respect to this pathogen. A study by Viswanathan and Kaur [55] reports the presence of *S. aureus* in 23% of a total of 120 samples from various vegetables in India. This incidence is attributed to postharvest and human contamination due to the management of the foods. These results make evident the permanence of the pathogen in this food group, the proper handling of Mexican producers, and the safety of their food.

5. Conclusion

The molecular techniques used in this study are suitable for the identification of *S. aureus* isolated from lettuce, increasing our capability of detecting this pathogen by improving the process and increasing the efficiency, contributing to the safety of this vegetable.

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