GENOTYPING AND HEMOLYTIC CHARACTERIZATION OF PATHOGENIC BACTERIA FROM SOME RAW AND COOKED FOODS

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ABSTRACT

This study aimed to identify pathogenic bacteria in contaminated food from cafeterias and restaurants at the University of Kerbala. Thirty-nine bacterial samples were collected from various foods, such as salads, falafel, and meat products (burger, kebab, and shawarma), before cooking. Bacteria were serially diluted, isolated on selective media, and identified based on biochemical characteristics, and 16S rDNA sequencing. Hemolysin production, seen in most bacteria from raw food samples, was determined using blood agar. Genomic DNA was extracted from all bacterial samples, and their 16S rDNA were analyzed through PCR, gene sequencing, and phylogenetic tree construction. Twenty-seven genetic variants representing both gram-positive and gram-negative bacteria were identified. Most of the bacterial isolates produced α or β hemolysin and are likely important causes of food poisoning. These results highlight the need for strict quality control in the cafeterias and restaurants at the university, improving the public's awareness of food safety issues, and possible routine medical examination of those who handle food at these locations.

Keywords: Contaminated food, Hemolysin, 16S rDNA, Phylogenetic tree, Genetic variation.

الخصائص الجينية والتحللية للبكتريا المرضية المعزولة من بعض الاغذية الخام والمطبوخة

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المستخلص

الهدف من هذه الدراسة التعرف على أنواع البكتيريا المسببة للأمراض الملوثة للأغذية المعزولة من بعض الكافتريات والمطاعم في جامعة كربلاء. اعتمدت الدراسة على 39 عينة تم جمعها من عدد من الأنواع الاغذية المختلفة مثل السلطة والفلافل ومنتجات اللحوم (بوركر والكباب والشاورما) قبل وبعد الطهي، تم عزل البكتيريا على اوساط انتقائية مختلفة بعد إجراء سلسلة من التخافيف العشرية. شخصت البكتيريا بالاعتماد الخصائص المظهرية وتتابع القواعد النتروجينية.قدرت قابلية البكتيريا على إنتاج السموم المحللة للدم وذلك بتنميتها على أجار الدم، وجدت ان معظم أنواع البكتريا المعزولة من عينات المواد الغذائية الخام تنتج هيموليسين ، استخلص الحامض النووي من جميع عينات البكتريا المنتجة وغير المنتجة لسم حال الدم hemolysinباستخدام عدة من شركة إنترون. دُرِس التغاير الوراثي للدنا المستخلص لجميع العينات من خلال تقنية سلسلة تفاعلات البلمرة PCR وتتابع النيوكليوتيدات لجين 16S rDNA والشجرة التطورية. حددت 27 نوع من البكتريا المتغايره وراثيا، وجد ان اغلب العزلات البكتيرية هي منتجة للـ α ، β هيموليسين وهذا يؤكد ان الوجبات الرئيسية كانت ملوثة ونتيجة لهذه الدراسة يجب تشديد الرقابة على الكافيتريات والمطاعم الجامعية ورفع مستوى الوعي للعاملين في الغذاءواجراء الفحوصات الطبية لهم بشكل دوري.

الكلمات المفتاحية:– الغذاء الملوث،انحلال الدم ، 16S rDNA ، الشجرة الجينية، التغاير الوراثي

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INTRODUCTION

Food is an excellent medium for growth and reproduction of microbes, making the bacteriological quality of both uncooked and cooked food important to consumers (18). Specifically, the extensive handling of crude sustenance, such as vegetables, increases the likelihood of contamination of these products by pathogens (34). In 2016, one study found an uncommon strain of *E. coli* O157:H7 in mixed salad that caused mild to bloody diarrhea and acute abdominal pain. The latest outbreak consisted of 161 cases, including 16 hospitalizations and two deaths (26). ====Foodborne illnesses or diseases are classified as intoxication ("food poisoning") or infectious depending on the specific cause of the illness. The causative agent must be present in sufficient numbers to cause symptoms, such as food contaminated with bacteria and/or bacterial toxins (35,15,20). For example, *Staphylococcus* spp. must reach 10^5 CFU per gram of food to yield enough toxin to cause emergence of symptoms (10). One important virulence factor produced by many pathogenic foodborne bacteria are hemolysins, a group of pore-forming toxins that destroy red cells and are often produced by *Staphylococcus aureus*. Hemolysins are classified as alpha, beta, gamma, delta, or epsilon based on their mechanism of action and effect on red blood cells (5,6,9,24,25,27) and are produced by both gram-positive and gram-negative bacteria. Microbiological investigations of food traditionally rely on identifying pathogenic organisms by culturing on selective media (e.g. Mannitol Salt Agar, *Salmonella-Shigella* Agar) and biochemical testing, but these approaches are often unwieldy and time-intensive (1,2,15,19). More recently, molecular methods for identifying pathogenic bacteria in foods have been adopted due to their rapidity and accuracy. In this approach, the polymerase chain reaction (PCR) amplifies 16S rDNA from samples of interest, which can then be sequenced for diagnostic purposes (4,13). The bacterial 16S rDNA gene has been widely used in phylogenetic studies because of its universality, conserved nature, and sufficient length for reliable sequence analysis (approximately 1500 bp) (23). In this study, we isolated bacteria from potentially contaminated foods, inferred their phylogeny based on 16S rDNA sequences, and determined their ability to produce hemolysins.

MATERIALS AND METHODS

Sample collection and culture-based identification:

Twenty-eight bacterial isolates were collected from raw and processed foods, including salads, falafel, and meat products (burger, kebab, and shawarma) before cooking in cafeterias and restaurants at the University of Kerbala between May and November 2016. Samples were stored and transported to the laboratory at 4°C. Bacteria were collected from the sample by scraping 11 g of food and adding this to 99 ml pepton water (0.1%), then mixing with a blender for 2 min before serially diluting the sample. From the final dilution of $10^{\wedge-3}$, 1 ml was added to duplicate petri dishes, then selective agar media at 45°C was added (e.g., MacConkey, Mannitol Salt, and *Salmonella-Shigella* agars) and the plates slowly moved for mixing. After the plates solidified, they were incubated at 37°C for 48 hours. After the incubation period, colonies were counted. Bacteria were isolated and tenatively identified based on their culture characteristics as previously described (8,14,16 and 19).

Determination of hemolysin production: Hemolytic activity of the bacterial samples was measured by culturing on blood agar prepared according to the manufacturer's instructions (Himedia). Briefly, the agar base was sterilized by autoclaving at 121°C for 15 min at 15 psi and cooled to 50°C before cattle blood was added to a final concentration of 5%. Bacteria were isolated by quadrant streaking, and plates were incubated at 37°C for 24 h. The presence and type of hemolysin(s) produced were determined as described previously (3).

DNA extraction

Genomic DNA was extracted from the bacterial samples using a total DNA G-spin iNtron kit (Korea) according to the manufacturer's instructions (11). DNA concentration and purity were determined by spectrophotometry at $A_{260/280}$.

Amplification of 16S Rdna

16S rDNA genes were PCR amplified using the universal primer set 8F: 5'-AGAGTTTGATCCTGGCTCAG-3' and U1492R: 5'-GTTACCTTGTTACGACTT-3'. Reactions also consisted of Maxime PCR premix (iNtron, Korea) 5 U/µl *Taq* polymerase, 2.5 mM dNTPs, 1X buffer, and 1X loading dye that was added to 2 µl DNA template and 10 pmol of each primer to a final volume of 16 µl. PCR was carried out with an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) at an initial denaturation of 94°C for 5 min, 35 cycles of 94°C for 45 sec, 62°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 5 min. Amplicons were then electrophoresed and visualized on a 2% agarose gel (11).

16S rDNA sequencing and alignment

Amplicon sequencing was performed by Macrogen (Canada) using the forward primer for each reaction. Sequences reaching 1500bp were scrutinized with FinchTV v.1.4.0 (Geospiza, Waltham, UK). Each 1500-bp amplicon was evaluated using a quality value, trimmed to 521 bp, and compared with sequences in the National Center for Biotechnology Information (NCBI) database with the BLASTN tool (www.ncbi.nlm.nih.gov) (36). A phylogenetic tree was constructed with MEGA v.6 (32) using parameters described by Saitou and Nei (31).

RESULTS AND DISCUSSIONS

16S rDNA was successfully amplified from 28 bacterial samples as shown in Fig. 1, similar to what has been found in other studies (23, 33).

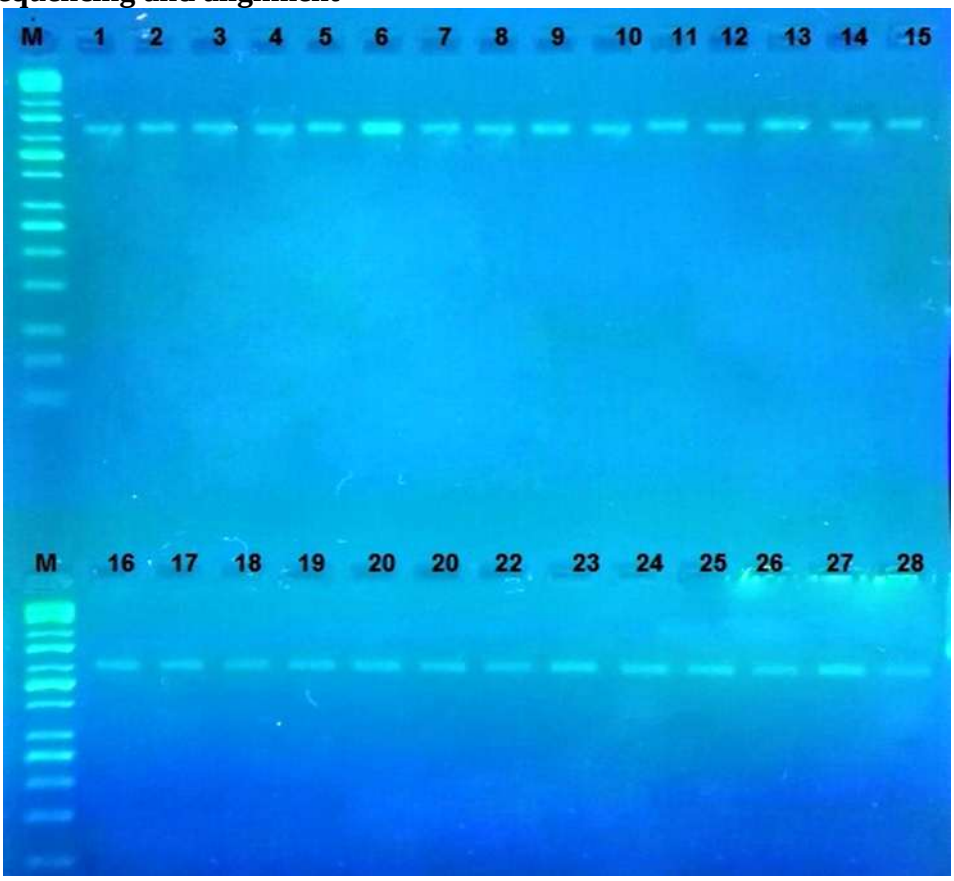

Figure 1. 16S rDNA amplicons of 28 bacterial samples collected from various food sources are shown. (M, marker)

The value of the 16S rDNA gene in taxonomic studies relies on its universality in both gram-negative and gram-positive bacteria (12,13). Moreover, sequencing the entire 16S rDNA gene can distinguish bacteria at the strain level (21,29,30).

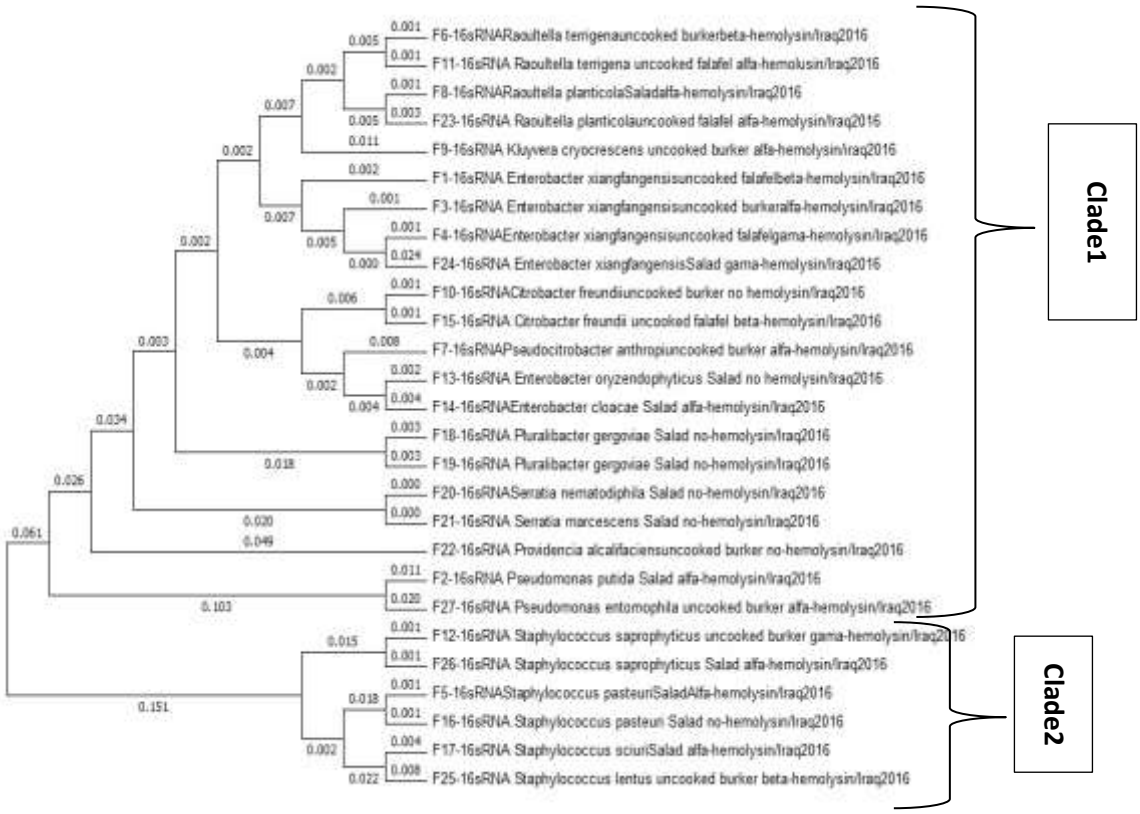

Figure 2. Neighbor-joining unrooted phylogenetic tree of bacterial 16S rDNA gene sequences from food samples in this study. Clade 1 and Clade 2 refer to gram-negative and gram-positive bacteria, respectively.

Figure 2 shows the putative neighbor-joining relationships between the bacteria isolated in our study based on their 16S rDNA homology with sequences in the NCBI database (31). One of the isolates yielded a poor-quality 16S rDNA sequence and was excluded from further analysis. The optimal tree had a branch length sum of 0.68765434, and evolutionary distances were calculated using the maximum likelihood method (33) and represent the number of base substitutions per site. Our phylogenetic analysis clustered the 27 isolates into monophyletic groups descending from ancestors such as F22 (*Providencia alcalifaciens* MG063180), F2 (*Pseudomonas putida* MG063164), and F27 (*Pseudomonas entomophila* MG063183) (7). Polyphyletic groups in Clade 1 (gram-negative bacteria) consist of various species that lack a common ancestor, including F6 (*Raoultella terrigena* MG063167), F4 (*Enterobacter xiangfangensis* MG063166), F10 (*Citrobacter freundii* MG063171), F13 (*Enterobacter oryzendophyticus* MG063173), F14 (*Enterobacter cloacae* MG063174), F18 (*Pluralibacter gergoviae* MG063176), F20 (*Serratia nematodiphila* MG063178), and F21 (*Serratia marcescens* MG063179). In contrast, Clade 2 includes paraphyletic groups of F26 (*Staphylococcus saprophyticus* MG063191), F5 (*Staphylococcus pasteuri* MG063186), F16 (*Staphylococcus pasteuri* MG063188), F17 (*Staphylococcus sciuri* MG063189), and F25 (*Staphylococcus lentus* MG063190). Isolates F12 (*Staphylococcus saprophyticus* MG063187) and F26 (*Staphylococcus saprophyticus* MG063191) are more closely related to each other than F5 (*Staphylococcus pasteuri* MG063186) and F16 (*Staphylococcus pasteuri* MG063188). 16S rDNA-based identification of pathogenic foodborne bacteria enables accurate diagnosis that can be accomplished by even small laboratories. An additional benefit of this genotyping method allows the classification of such bacteria into phylogenetic groups to study their relationships with each other. Moreover, the impact of environmental conditions on bacterial evolution and (sub)speciation can be inferred by identifying polymorphisms in the organisms' 16S rDNA genes (11,28).

Table 1. Homology of gram-positive bacterial 16S rDNA sequences from our study with sequences from the NCBI database.

Sequencing ID	Accession no.	Putative strain	Top BLASTN hit	Food source	Type of toxin	Identity %	e-value	Range
F5	MG063186	*Staphylococcus pasteuri*	*Staphylococcus pasteuri* ATCC 51129	Salad	alpha hemolysin	100%	0.0	26-546
F12	MG063187	*Staphylococcus saprophyticus*	*Staphylococcus saprophyticus* ATCC 15305	Uncooked burger	gamma hemolysin	99%	0.0	78-597
F16	MG063188	*Staphylococcus pasteuri*	*Staphylococcus pasteuri* ATCC 51129	Salad	gamma hemolysin	100%	0.0	28-548
F17	MG063189	*Staphylococcus sciuri*	*Staphylococcus sciuri* DSM 20345	Salad	alpha hemolysin	99%	0.0	430-950
F25	MG063190	*Staphylococcus lentus*	*Staphylococcus lentus* MAFF 911385	Uncooked burger	beta hemolysin	100%	0.0	60-580
F26	MG063191	*Staphylococcus saprophyticus*	*Staphylococcus saprophyticus* ATCC 15305	Salad	alpha hemolysin	100%	0.0	430-950

Table 2. Homology of gram-negative bacterial 16S rDNA sequences from our study with sequences from the NCBI database.

Sequencing ID	Accession no.	Putative strain	Top BLASTN hit	Food source	Type of toxin	Identity%	e-value	Range
F1	MG063163	*Enterobacter xiangfangensis*	*Enterobacter xiangfangensis*	Uncooked falafel	beta hemolysin	100%	0.0	21-541
F2	MG063164	*Pseudomonas putida*	*Pseudomonas putida* NBRC 14164	Uncooked falafel	gamma hemolysin	99%	0.0	63-576
F3	MG063165	*Enterobacter xiangfangensis*	*Enterobacter xiangfangensis*	Uncooked burger	alpha hemolysin	99%	0.0	19-838
F4	MG063166	*Enterobacter xiangfangensis*	*Enterobacter xiangfangensis* 10-17	Uncooked falafel	gamma hemolysin	99%	0.0	20-540
F6	MG063167	*Raoultella terrigena*	*Raoultella terrigena* NBRC 14941	Uncooked burger	beta hemolysin	99%	0.0	57-577
F7	MG063168	*Pseudocitrobacter anthropic*	*Pseudocitrobacter anthropi* C138	Uncooked burger	alpha hemolysin	99%	0.0	26-543
F8	MG063169	*Raoultella planticola*	*Raoultella planticola* ATCC 33531	Salad	alpha hemolysin	99%	0.0	69-589
F9	MG063170	*Kluyvera cryocrescens*	*Kluyvera cryocrescens* NBRC 102467	Uncooked burger	alpha hemolysin	99%	0.0	54-573
F10	MG063171	*Citrobacter freundii*	*Citrobacter freundii* LMG 3246	Uncooked burger	gamma hemolysin	99%	0.0	02-522
F11	MG063172	*Raoultella terrigena*	*Raoultella terrigena* NBRC 14941	Uncooked falafel	alpha hemolysin	99%	0.0	52-571
F13	MG063173	*Enterobacter oryzendophyticus*	*Enterobacter oryzendophyticus* REICA_082	Salad	gamma hemolysin	100%	0.0	21-541
F14	MG063174	*Enterobacter cloacae*	*Enterobacter cloacae* ATCC 13047	Salad	alpha hemolysin	99%	0.0	82-603
F15	MG063175	*Citrobacter freundii*	*Citrobacter freundii* LMG 3246	Uncooked falafel	beta hemolysin	99%	0.0	06-526
F18	MG063176	*Pluralibacter*	*Pluralibacter*	Salad	gamma	99%	0.0	49-563

		gergoviae	***gergoviae* JCM1234**		**hemolysin**			
F19	**MG063177**	***Pluralibacter gergoviae***	***Pluralibacter gergoviae* JCM1234**	**Salad**	**gamma hemolysin**	**99%**	**0.0**	**47-565**
F20	**MG063178**	***Serratia nematodiphila***	***Serratia nematodiphila* DZ0503SBS1**	**Salad**	**gamma hemolysin**	**100%**	**0.0**	**73-593**
F21	**MG063179**	***Serratia marcescens***	***Serratia marcescens* NBRC 102204**	**Salad**	**gamma hemolysin**	**99%**	**0.0**	**55-575**
F22	**MG063180**	***Providencia alcalifaciens***	***Providencia alcalifaciens* NCTC 10286**	**Uncooked burger**	**gamma hemolysin**	**100%**	**0.0**	**61-581**
F23	**MG063181**	***Raoultella planticola***	***Raoultella planticola* ATCC 33531**	**Uncooked falafel**	**alpha hemolysin**	**99%**	**0.0**	**66-586**
F24	**MG063182**	***Enterobacter xiangfangensis***	***Enterobacter xiangfangensis* 10-17**	**Salad**	**gamma hemolysin**	**97%**	**0.0**	**24-546**
F27	**MG063183**	***Pseudomonas entomophila***	***Pseudomonas entomophila* L48**	**Uncooked burger**	**alpha hemolysin**	**98%**	**0.0**	**76-596**

Tables 1 and 2 summarize the 16S rDNA-based genotypes and hemolytic capabiliteis of the 27 bacterial isolates analyzed in our study. Bacteria from various food sources, including salad, uncooked falafel, and uncooked burger, produced hemolysins, which supports the findings of other foodborne illness-related studies (3,17,28). The expected value, which is an estimate of how often you expect to see the same similarity by chance, was zero for all of the bacterial isolates (Table 1 and Table 2). The high score (bits), which is a statistical scale that reflects the similarity between the experimental sequences and sequence data in genbank, indicated a high degree of similarity. Our 16S rDNA gene isolate data matched genbank accession ID numbers SUB3114013 and SUB 3113900, with high but varying conformity (97-100%) for all isolates. Our results corroborate those from a similar study (8) that found various pathogeneic bacterial isolates by 16S rDNA gene PCR in diffierent kinds of food (11). Workers' hygeine, food storage methods, and cooking and storage temperatures are major contributors to contamination. Therefore, personal and environmental hygiene are essential to avoid foodborne illness. The results of this study show that uncooked food was more susceptible to contamination due to the inappropriate storage locations and temperatures, which led to increased contamination of food which could result in food poisoning. One key factor is to avoid allowing food to remain in the critical "danger zone" from 40 to 140°F any longer than necessary. In conclusion, To ensure the safety of fresh foods, measures must be taken to limit their contamination by pathogenic bacteria, especially those with hemolytic activity. This genomic study used 16SrDNA to identify a variety of bacterial isolates, which provides novel information about the genomic characteristics and pathogenesis mechanisms of bacteria isolated from food. Moreover, this genomic information would be beneficial for elaboration of innovative biocontrol approches to prevent the pathogenesis of such bacterial strains.

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