Molecular detection of Erwinia herbicola isolated from Nuts

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Abstract

Food borne pathogens are major impediment of the public health and economic development, mostly Enterobacteriaceae is inherent foodborne pathogens of which *E. herbicola* have recently emerged significant in ready eat food. The aim of this study was to determine quantitatively of viable bacterial isolates and molecular diagnosis of *Erwinia herbicola* by SYBR Green real-time PCR in nuts. The bacterial species isolates of nuts are Coagulase + *Staphylococcus*, *E. coli*, *Enterobacter cloacae* and *Erwinia herbicola*. The highest rate of Coagulase + *Staphylococcus* species of two hazelnuts and two cashew samples and *Erwinia herbicola* of one almond sample, theses bacterial isolates from nuts were present between microbiological unsatisfactory and marginal levels, however in present study selected *E. herbicola* to molecular identification by used SYBR Green qPCR assay to amplify the rep gene. The specificity of assay was represented molecular quantitation correlation was between ct values and enumeration on aerobic plate agar count of bacteria in nuts, the results showed that assay is specific diagnosis of *E. herbicola* through ct values ranging from 20.5.to 23.6 for four samples.

Key words: Enterobacteriaceae; SYBR Green qPCR; Staphylococcus

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Introduction

Food safety is burden of safe food and policy appropriate for detection and prevention of food borne pathogens to ensure safety and quality of food to customers; globally, it is through U.S. Food and Drug Administration (FDA) and Institute of Food Technologists (IFT), In 2011, Center for Disease Control and Prevention (CDC, U.S) reported that is approximately 9.4 million of human food borne diseases which includes 1351 death cases per year [1]. Bacteriological profile is important scale in food safety for understanding of how can control them and pass food safely to the consumer, Erwinia herbicola is considered as contaminated bacteria of nuts.

This study found that most of nut products are not subject to instructions of food safety [2] and sold in unpackaged storage and unknown trademark. National studies of bacterial prevalence in nut products were rare, and few global studies interested with E. herbicola considered questioned pathogen to human health. E. herbicola is Gram-negative bacterium that belongs to the family Enterobacteriaceae. It has several names Enterobacter agglomerans or Pantoea agglomerans which caused some confusion in finding pathogenity of human and animals. However, there are many studies extended more than 40 years showed clinically significant of E. herbicola is associated with gastroesophageal reflux disease caused to bacteremia [3], also Cruz study in 2007 isolated E.herbicola of blood, urine, abscesses, and thorax culture which are suffering from bacteremia [4] and in Clinical report of young 18 years suffered from scalp abscess due to E. herbicola [5]. The E. herbicola is considered an allergen as the consumption of grains contaminated grains causes to hypersensitivity pneumonitis [6] and also, dust borne E. herbicola cause to bronchial hyperreactivity [7].

Patients and Methods

Setting the samples

Five 2 Oz subs of nut products from previous days to months' production, according to information obtained from the store owner (8). Total Samples were 100 of various not canned nuts that comprised 20 of each walnut, almonds, cashew nuts, pistachio and hazelnuts, which were randomly purchased from different stores and hawkers in Al-Diwaynia city (table 1). The samples were transported to the Microbiology laboratory under sterile conditions and sequentially, un-shell and shelled nut meats be separated, cleaned in sterile sampling bags and grinding before further bacterial isolation.

Bacteriological isolation:

Primary bacterial isolation performed on non-selective media, which included blood agar and differential MacConkey and XLD agar. This study was limited to bacteria isolation, so that utilized the cycloheximide concentration of 100 µg/ml added to culture agar to inhibit the growth of yeasts and fungi may be associated with nut meat samples (9). In order to diagnosis of *E. herbicola* and other bacterial species performed using the biochemical tests.

Bacterial enumeration:

One gram of the crushed nutmeat sample was suspended in 10 ml of sterile pepton water, for the purpose of the work the serial dilutions of the samples 0.1 in triplicate, in order to do 10-fold dilution of the sample. The full volume of each dilution is spread on nutrient agar plates incubated at 37 °C for 24 hours enough to observe the growth of bacterial isolates which were counted by the digital colony counter. For the bacterial counts were estimated

as colony forming units (cfu) per ml. CFU in one nut meat is equal multiplied by the volume of diluent (10). Analysis of aerobic count (APC) to microbiological satisfactory, marginal, unsatisfactory and potentially hazardous quality levels, according into Food Standards Australia New Zealand guideline determination of the ready eat food [11].

Bacterial Strains and DNA Extraction:

The bacterial DNA extraction was performed by bacteria DNA isolation kit (Geneaid Biotech, Taiwan) of *E. herbicola* grow overnight culture to microfuge tube suspended with 500 µl of sterile distill water and spin at 14000 rpm for 1 minute, then next steps used pellet according into manual instructions. The bacterial genome is analyzed by agarose 0.75% electrophoresis 100 vol. for 15 min. Storage of samples at -20ºC for PCR assay.

SYBR Green Based Real-Time PCR Assay:

PCR amplification was performed in 200 µl tubes which contains 25µl GoTaq qPCR master mix (Promega, USA), 0.5 µM of each upstream PCR primer rep-F ´5 TTGTGGGGG ACATATAACC-3´ and downstream rep-R ´5-AGGGCCATAGTGAGGAAGGT-3´ [12] and 5µl genomic template and up to final volume 40µl of nuclease-free water. The negative control reaction of amplification was conducted in 5µl of nuclease-free water instead of genomic template. The SYBR Green based real-time PCR assay was determined using Mx3000Pro (Agilent Technologies, Germany) in touch program: hot-start activation 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds and dissociation step of 60–95°C. The 10-fold diluents of *E. herbicola* into amplified rep gene, which regarded as standards to relative quantition of bacterial load in order 10, 10^2, 10^3, 10^4 and 10^5 series. For standard curve was performed in same qPCR reaction volumes in getting on the relation between ct values to cfu/g [13].

Results and discussion

Nuts such as walnut, almonds, cashew nuts, pistachio and hazelnuts of the food products are mostly consumed as snack meals in our country and be sold in different places, including kiosks and stores prone to direct environmental conditions, which become more exposed to bacterial pollution depending on the factors storage method and duration of storage and obligation to sellers in hygiene guidance that affect the quality, taste and health of the product. A total of 100 nut meat samples were collected from 20 different locations in the city (9 stores and 6 koisks- Hawkers). All samples that investigated were classified by storage period, type of packaging and source of sample purchase table (1).

Table 1.

Distribution of nut samples by storage period, type of packaging, source and location of sample purchase.

Sample Description	No. samples	Storage period (week)	Type of Packaging	Purchase source	Location of purchase
Walnut,Un shelled	20	2 - 16	Unpackaged	Hawker and store	Diawynia
Almonds,Un shelled	20	1-12	Unpackaged	Hawker and store	Diawynia
Cashew, Un shelled	20	2- 14	Unpackaged	Hawker and store	Diawynia
Pistachio,Un shelled	20	4- 12	Unpackaged	Hawker and store	Diawynia
Hazelnut, shelled	20	3- 15	Unpackaged	Hawker and store	Diawynia

In order to differentiate of bacteria isolated from nuts, the Gram positive bacteria were tested for catalase and coagulase test, which only gave us results to coagulase + *staphylococcus spp.*, while the Gram negative bacteria followed the tests in table (2).

Table 2.

Biochemical reactions of Gram negative bacteria for the diagnosis.

Test	*E. coli*	*E. cloacae*	*E.herbicola*
Indol production	+	_	(–)
Methy red test	+	_	V
Voges-Proskauer test	–	+	V
Citrate utilization	–	+	V
Urease	–	+	(–)
Phenylalanine deaminase	–	_	(–)
Hydrogen sulfide	–	–	–
ONPG	+	+	+
Sucrose	(+)	+	(+)
Lactose	+	_	V
Sorbitol	V	+	V
Mannitol	+	+	+
Inositol	–	V	(–)
Lysine hydrolyse	(+)	_	–
Ornithine hydrolyse	(+)	+	–
Motility test	(+)	+	(+)
Yellow pigment	_	–	(+)
The chemical reactions : + represent 95 – 100% positive; (+) represent 75-89% positive; V 25-74% positive;(-): 10-24% positive; – 0-9%positive .			

The Standard method is used in this study for the presence of bacterial species in nut samples for ready to eat foods, it is important to validated results according to Food and Drug Administration guidelines [8].

FSANZ guideline determination of the microbiological quality of food products depending on aerobic plate count (APC), the highest rate of Coagulase + *Staphylococcus* species in two samples of hazelnuts in ranged 5.3 $\dot{X}$ 10^3 cfu/g and 2$\dot{X}$ 10^3 cfu/g in two samples of cashew were microbiological unsatisfactory quality. *E.coli* growth was detected in ranged 2$\dot{X}$ 10^2 cfu/g of three samples of walnut, this count is greater than 100 cfu/g unsatisfactory quality level. This study recorded that one sample of walnut, three of cashew and one of hazelnuts in ranging 8$\dot{X}$ 10^2 to 4.1$\dot{X}$ 10^2 cfu/g of *Enterobacter cloacae*, which are within the microbiological marginal quality values (10^2-10^4 cfu/g). The extracted values of one samples of each walnut, almond, cashew and hazelnut was 10^3 to 5.4 $\dot{X}$ 10^2 cfu/g of *Erwinia herbicola* within the microbiological marginal scale of Enterobacteriaceae, while in pistachioa was less than 5 cfu/g [11] as shown in figure (1). There have been no reported foodborne outbreaks related to consuming of nut meat registered in Iraqi public health, which is consistent with the results of this study that did not report the microbiological potentially hazardous quality levels. Previous study only included prevalence of that isolated bacteria: *Escherichia coli*, *Erwinia spp* and *E. cloacae* from nut samples in Iraqi markets [12].

All samples of nut meats in this study were negative for Salmonella and shigella species grow on XLD agar for 48 hours, that compared to in previous study showed that unsatisfactory values of *Salmonella* spp. and *E. coli* has been isolated from nut and seed products in United Kingdom (14). According to the results of this study, storage periods of nuts was important factor in all positive samples of unsatisfactory quality has affected the rate of APC for more than 10 weeks and compared to previous studies [10].

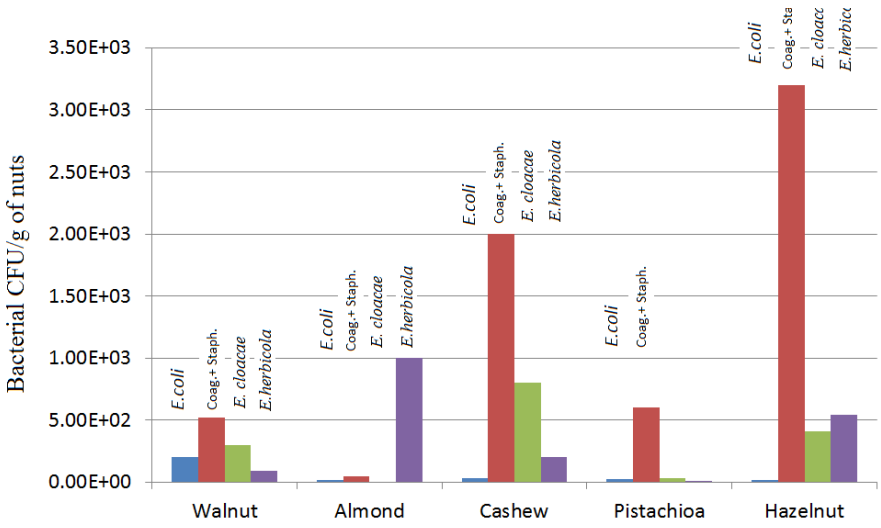

Figure1.

Bacteriological profile of walnut, almond, cashew, pistachio and hazelnut in cfu/g.

Erwinia herbicola belongs to Enterobacteriaceae and classified within opportunistic bacteria of humans and animals [5], but there are many studies reported *Erwinia herbicola* consider to be clinically significant such as bacteremia and skin infections to be questioned between pathogencity and inexactly taxonomic identification [4]. So this study that postulated the molecular diagnosis of *E. herbicola* by SYBR Green–based qPCR assay is specific, precise and sensitive DNA quantification as shown in figure (2). The SYBR Green qPCR E. herbicola diagnosis was for four samples of 9.2 $\dot{X}10$ walnut, 10^3 almonds, 2 $\dot{X}10^2$ cashew and 5.4 $\dot{X}10^2$ cfu/g hazelnut, but bacterial load less than 5 cfu/g in pistachio could not detect. The bacterial contamination probabilities of nuts are accompanying to unhygienic handling or machines in industrial cracking and shelling processes. Therefore, nuts contamination could become of potential hazard if the nuts are contaminated *with E. herbicola* growth during industrial processes and long storage [15].

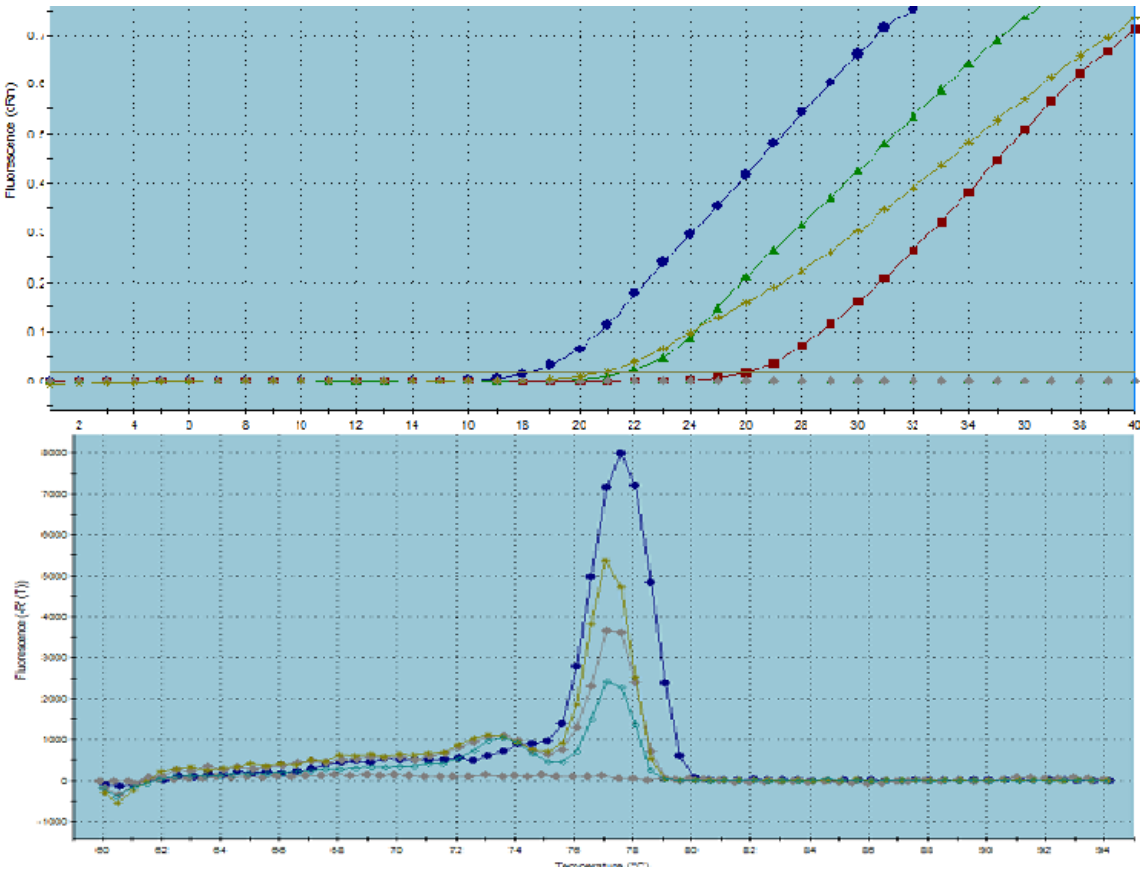

Figure 2.

Amplification plot and dissociation curve of positive four samples of *E. herbicola*.

Primers were selected to amplify the rep gene of *E. herbicola.* The specificity of primers were used in this study for sybr green qPCR assay represents good correlation (RSq:0.998) was between ct values and enumeration on aerobic plate agar count (APC), the results showed that assay is specific diagnosis of *E. herbicola*. The ct values ranging from 20.5.to 23.6 for four samples table (3). The melting temperature calculated at the end of each sybr green q PCR assay was 77.7 °C that was uniformed for all four positive sample of *E. herbicola*. There are several factors may be effect on determination of gene target by real-time PCR in nuts that includes the low yield of pathogenic genome extraction and inhibitor of polymerase lead to false negative or low efficiency of amplification product, so the present study found dramatically differences of amplification qPCR plots in positive three samples of *E. herbicola* in three samples walnuts, almond, cashew and hazelnut [16].

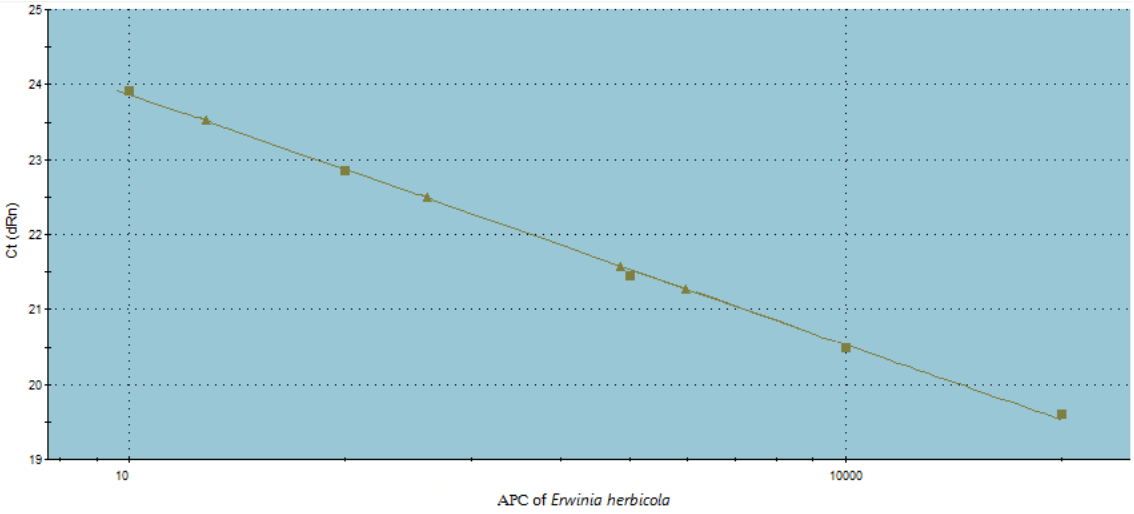

Figure 3.

Correlation between aerobic plate count (CFU/g) of E. herbicola and t values determined by Sybr green real-time PCR of positive 4 samples of nuts.

The range of 5 orders to linear quantifications were reported in amplification efficiency 98.2 to 101.4% obtained from the enumeration of bacteria in nuts in this study, which compared amplification efficiency of 88 to 115% indicates in several previous studies used PCR assay obtained to linear quantification relation of seed and fruit food borne bacteria by be enumerated 10 fold serial dilutions (CFU/g)of bacterial culture and ct values which indicates specific quantification of amplification efficiency of greater than 96% and R^2 of 0.998 [17,18].

Table 3.
The bacterial plate count (cfu/g) and real-time PCR ct values of *E. herbicola* in nuts and their amplification efficiency.

Sample	Ct value	Cfu/g count	Amplification efficiency (%)
Almond	20.5	10^3	99.5
Hazelnut	21.6	$5.4\dot{X}\ 10^2$	101.4
Cashew	22.5	$2\dot{X}\ 10^2$	98.2
Walnut	23.6	$9\dot{X}\ 10$	99.8

The results concluded in this study indicate that nuts sold in Kiosks were more exposed to bacterial contamination of Enterobacteraceae and Coagulase + *Staphylococcus* , has been identified numeration by conventional plate count. These bacterial isolates from nuts present between microbiological marginal and unsatisfactory levels, however in present study *E.*

herbicola selected to molecular quantitation in linear correlation between bacterial count and Ct values .

Bacterial load less than 5 cfu/g of in pistachio sample did not detected by SYBR Green qPCR assay and disappeared in standard curve, despite the primer specifically and amplification efficiency in this study, it proves that there are isolates of *E. herbicola* that need to extend of specific molecular diagnosis, so requiring further studies include DNA sequencing of rep gene and genotyping *E. herbicola* isolated from nuts and other fruits [19]. However, the transition of microorganism from unknown to known food born pathogen require research data the targeted knowledge of pathogenic virulence, taxonomic identification, and genotypes of pathogen [20-23].

Ethical Approval

The study was approved by the Ethical Committee.

Conflicts of Interest

The author declare that she has no competing interests.

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