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Prevalence of foodborne pathogens in meat samples in Palestine

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Abstract

Foodborne diseases occur worldwide, including those acquired through the consumption of contaminated meat. This study aimed to investigate the prevalence of enterotoxigenic *Staphylococcus aureus, Salmonella* and *Escherichia coli* pathotypes in different meat types. Forty meat samples fresh (n=35) and frozen (n=5) were purchased from local markets in Jenin district, Palestine. Multiplex PCR was used to detect enterotoxigenic *S. aureus, Salmonella* and *E. coli* pathotypes. Total mesophilic aerobic bacterial count ranged between 4.3 $\log_{10}$ to 5.7 $\log_{10}$ cfu/g for frozen meat and 6.95 $\log_{10}$ to 7.78 $\log_{10}$ cfu/g for fresh meat. The prevalence of *S. aureus, Salmonella* and *E. coli* was 30%, 25% and 95%, respectively. Among tested *S. aureus* strains 75% were enterotoxigenic. Two other samples of non *S. aureus (FemA$^-$)* were enterotoxigenic; one was *sec*+ and the other was *see*+. The results also showed that 89.5% of meat samples contaminated with *E. coli* that belonge to enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), diffuse adherent *E. coli* (DAEC) pathotypes. According to these results, it is recommended to establish a suitable surveillance program for microbial contamination with all foodborne pathogens.

Introduction

Food is considered the most important energy source for humans and animals. Meat may be easily contaminated with different pathogens if not handled appropriately (Mead *et al*., 1999). The World Health Organization (WHO) defines foodborne illnesses as diseases, usually either toxic or infectious in nature, caused by agents that enter the human body through the process of food ingestion. There are more than 200 known causative agents can cause foodborne diseases; these include bacteria, parasites, viruses, prions, toxins and metals. The symptoms and severity of foodborne illnesses vary, range from mild gastroenteritis to life-threatening neurologic, hepatic, and renal syndromes (Mead *et al*., 1999). In 2005, WHO reported that 1.8 million people died from diarrheal diseases and a high proportion of these cases due to contamination of food and drinking water (WHO, 2008). Although large number of bacterial strains have been identified to be involved in foodborne diseases, many other new emerging strains also reported (WHO, 2008). In developed counties, the annual incidence of microbiological foodborne illnesses is estimated to be around 30% of the population (De Guisti *et al*., 2007).

In Palestine, a total of 250 stool samples were collected during an outbreak from symptomatic and asymptomatic patients in northern Palestine in 1999. A total of 176 (70.4%) were identified as Shiga toxigenic *E. coli* (STEC), of the 176 STEC isolates, 124 (70.5%) were of serotype O157 (Adwan *et al*., 2002). Another study on raw beef samples reported that 14.4% of samples were contaminated with STEC (Adwan and Adwan, 2004). Enterotoxigenic *S. aureus* strains were also reported in raw milk of clinically healthy sheep and cows (Adwan *et al*., 2005). Another study on an outbreak of acute gastroenteritis and diarrhea among children in Gaza strip showed that various enteropathogens including; *Shigella* spp, *Campylobacter coli/jejuni*, *E. coli* O157:H7 and *Salmonella* spp were identified using conventional and molecular techniques (Abu Elamreen *et al.*, 2007). This study aimed to detect enterotoxigenic *S. aureus* (ETSA), *Salmonella* spp and *E. coli* pathotypes from meat samples using PCR technique and to estimate the level of bacterial contamination in these samples.

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Table 1. Target genes for PCR amplification, amplicon size, primer sequences and annealing temperature

Organism	Target Gene	Oligonucleotide sequence (5? 3)	Amplicon Size (bp)	Annealing temperature	Reference	Primer mix
E. coli	*Mdh*	ACT GAA AGG CAA ACA GCC AGG C CGT TCT GTT CAA ATG CGC TCA GG	392	59°C	Hsu *et al*., 2006	1
Salmonella spp.	*HindIII DNA fragment*	GTCACGGAAGAAGAGAAATCCGTACG GGGAGTCCAGGTTGACGGAAAATTT	375	56°C	Tsen *et al*., 1994	2
S. aureus	*FemA*	TATGAGTTAAAGCTTGCTGAAGGTT TTACCAGCATTACCTGTAATCTCG	296	56°C	Kawasaki *et al*., 2012	2
ETSA	*sea*	CCTTTGGAAACGGTTAAAACG TCTGAACCTTCCCATCAAAAAC	127	55°C	Becker *et al*., 1998	3
ETSA	*seb*	TCGCATCAAACTGACAAACG GCAGGTACTCTATAAGTGCCTGC	477	55°C	Becker *et al*., 1998	3
ETSA	*sec*	CTCAAGAACTAGACATAAAAGCTAGG TCAAAATCGGATTAACATTATCC	271	55°C	Becker *et al*., 1998	3
ETSA	*sed*	CTAGTTTGGTAATATCTCCTTTAAACG TTAATGCTATATCTTATAGGGTAAACATC	319	55°C	Becker *et al*., 1998	3
ETSA	*see*	CAGTACCTATAGATAAAGTTAAAACAAGC TAACTTACCGTGGACCCTTC	178	55°C	Becker *et al*., 1998	3
EHEC	*VT*	GAGCGAAATAATTTATATGTG TGATGATGGCAATTCAGTAT	518	59°C	Gómez-Duarte *et al*., 2009	4
EHEC, EPEC	eae	CTGAACGGCGATTACGCGAA CGAGACGATACGATCCAG	917	59°C	Gómez-Duarte *et al*., 2009	4
EPEC	*bfpA*	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	326	59°C	Gómez-Duarte *et al*., 2009	4
EAEC	*aggR*	GTATACACAAAAGAAGGAAGC ACAGAATCGTCAGCATCAGC	254	59°C	Gómez-Duarte *et al*., 2009	4
ETEC	*LT*	GCACACGGAGCTCCTCAGTC TCCTTCATCCTTTCAATGGCTTT	218	59°C	Gómez-Duarte *et al*., 2009	5
ETEC	*ST*	GCTAAACCAGTAGAG(C)TCTTCAAAA CCCGGTACAG(A)GCAGGATTACAACA	147	59°C	Gómez-Duarte *et al*., 2009	5
DAEC	*daaE*	GAACGTTGGTTAATGTGGGGTAA TATTCACCGGTCGGTTATCAGT	542	59°C	Gómez-Duarte *et al*., 2009	5

Materials and Methods

Collection of samples

Forty meat samples were purchased randomly during May-June 2014, from different localities in Jenin district. These included 35 fresh samples (13 beef, 13 chicken and 9 turkey) and 5 frozen samples (2 beef, 2 chicken and 1 turkey). Samples were transferred under aseptic conditions to the Microbiology Laboratory, Department of Biology at An-Najah National University-Nablus, Palestine.

Sample preparation and bacterial culturing

A meat sample of 10 g was homogenized in 90 ml Tryptone Soya Broth-Yeast Extarct (TSBYE) medium. Six of serial decimal dilutions of sample with sterile normal saline were cultured in duplicates on nutrient agar. The plates were then incubated at 37°C for 24 h before colonies were counted. At the same time, 5 ml of TSBYE was incubated at 37°C/18-24 h and used for DNA extraction. The same sample subcultured on Xylose-Lysine Deoxycholate (XLDA), Mannitol salt agar (MSA) and MacConkey for further confirmation.

DNA extraction

DNA was prepared for PCR according to the method described previously with some modifications (Adwan *et al*., 2013). Briefly, 1.5 ml from overnight TSBYE broth was centrifuged for each DNA extraction preparation, the pellet washed twice with 1 ml of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The pellet was then resuspended in 0.5 ml of sterile distilled H_2O, and boiled for 10-15 min. The cells then immediately were incubated on ice for 10 min. The debris was pelleted by centrifugation at 11,500 × g for 5 min. DNA concentration was determined using a spectrophotometer and DNA samples were stored at -20ºC until use.

Detection of S. aureus *and* Salmonella *spp.*

The used primers targeted *Salmonella* species-specific 1.8 kb HindIII DNA fragment sequence and *S. aureus fem*A gene are shown in Table 1. (Tsen *et al.* 1994; Kawasaki *et al*., 2012). PCR reaction mix (25 µL) was performed using 12.5 µL of PCR premix with $MgCl_2$ (ReadyMixTM Taq PCR Reaction Mix with $MgCl_2$, Sigma), 0.4 µM of each primer, and 2 µL DNA template. DNA amplification was performed using thermal cycler (Mastercycler Personal, Eppendorf) according to the following conditions: initial denaturation for 2 min at 94°C followed by 40 cycles at 94°C for 20 sec for denaturation, annealing at 56°C for 30 sec and extension at 72°C for 30 sec. Final extension was carried out at 72°C for 5 min. The amplified products were examined by 2% agarose gel electrophoresis. A DNA ladder of 100 bp was also included in all gels (100 bp DNA ladder RTU, GeneDireX).

Detection of staphylococcal enterotoxin (sea-see) genes

Primer nucleotide sequences and expected sizes of amplicons for staphylococcal enterotoxin genes *sea, seb, sec, sed* and *see* are presented in Table 1 (Becker *et al.* 1998). The PCR reaction mix and

Table 2. Distribution of *Salmonella* spp. and *S. aureus* in different meat samples using PCR

Source	No. of samples	Microorganism		
		Salmonella spp. (*Hind III DNA fragment*$^-$)	*S. aureus* (*FemA*$^-$)	*Salmonella* spp. and *S. aureus* (*FemA*$^+$+*Hind III DNA fragment*)
Beef	(n=15)	1 (6.6%)	5 (33.3%)	0(0%)
Chicken	(n=15)	4 (26.6%)	2 (13.3%)	2(13.3%)
Turkey meat	(n=10)	2 (20%)	2 (20%)	1(10%)
Total %	(n=40)	7 (17.5%)	9 (22.5%)	3(7.5%)

Table 3. Distribution of staphylococcal enterotoxin genes *(sea-see)* in meat samples

			staphylococcal enterotoxin genes					
Source	No. of samples	*S. aureus* (*FemA*$^+$ gene)	*sea*$^+$	*Seb*$^+$	*sec*$^+$	*sed*$^+$	*see*$^+$	*sea*$^+$ + *see*$^+$
Beef	(n=15)	33.3% (5/15)	20% (1/5)	0% (0/5)	20% (1/5)	20% (1/5)	20% (1/5)	20% (1/5)
Chicken	(n=15)	26.7% (4/15)	25% (1/4)	0% (0/4)	0% (0/4)	0% (0/4)	50% (2/4)	25% (1/4)
Turkey meat	(n=10)	30% (3/10)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)	0% (0/3)
Total %	n=40	30% (12/40)	16.7% (2/12)	0% (0/12)	8.3% (1/12)	8.3% (1/12)	25% (3/12)	16.7% (2/12)

detection of amplified fragments were carried out as above. DNA amplification was performed using thermal cycler as follows: initial denaturation for 2 min at 94°C followed by 30 cycles at 94°C for 1min for denaturation, annealing at 55°C for 1 min and extension at 72°C for 2 min. Final extension was carried out at 72°C for 5 min.

Detection of E. coli *mdh gene*

E. coli was identified by PCR with specific primers for malate dehydrognase gene *(mdh)* as described previously (Hsu *et al.* 2006). Primer nucleotide sequences and expected size of amplicon are presented in Table 1. The PCR reaction mix and detection of amplified fragments were carried out as above. DNA amplification was performed as follows: initial denaturation for 2 min at 94°C followed by 30 cycles at 94°C for 1 min for denaturation, annealing at 59°C for 30 sec and extension at 72°C for 1 min. Final extension was carried out at 72°C for 5 min.

Detection of E. coli *pathotypes*

The targeted genes for *E. coli* pathotypes included enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC) and diffusely adherent *E. coli* (DAEC) were amplified using oligonucleotide primer pairs. The primers and expected sizes of amplicons are listed in Table 1 (Gómez-Duarte *et al.*, 2009). PCR reaction mix and detection of amplified fragments were carried out as above. DNA amplification was performed as follows: initial denaturation for 2min at 94°C followed by 40 cycles at 92°C for 30 sec for denaturation, annealing at 59°C for 30 sec and extension at 72°C for 30 sec. Final extension was carried out at 72°C for 5 min.

Results

Estimation of bacterial level of contamination

The total aerobic bacterial count ranged between 4.3 $\log_{10}$ to 5.7 $\log_{10}$ cfu/g for frozen meat and 6.95 $\log_{10}$ to 7.78 $\log_{10}$ cfu/g for fresh meat. MacConkey agar showed that 95% of samples were lactose fermenter with bright pink color colonies. XLD agar showed that, 22.5% of samples had colonies with black centers. Results also showed that 65% of the subcultured samples on MSA were mannitol fermenters.

Detection of Salmonella *spp.,* S. aureus *and ETSA*

The prevalence of *Salmonella* spp., *S. aureus* and both in studied meat samples was 17.5% and 22.5% and 7.5%, respectively. The distribution of these pathogenes in meat types are shown in Table 2. The prevalence of staphylococcal enterototoxin genes among *S. aureus* isolates was 16.7%, 0.0%, 8.3%, 8.3% and 25% for *sea, seb, sec, sed* and *see*, respectively. *Sea* and *see* were found in combination in 16.7% of the studied samples. Two beef meat samples, *FemA*$^-$ and non mannitol fermenter; one carried sec and the other carried see were detected.

Table 4. Prevalence of uni-infected *E. coli* pathotypes in studied meat samples

Source	No. of samples	*E. coli* pathotypes (Uni-infection)						
		EHEC ($VT^+ + eae^+$)	EPEC($bfp A^+ + eae^+$)	EAEC ($aggR^+$)	DAEC ($daaE^+$)	ETEC		
						LT^+	ST^+	LT^+ and ST^+
Beef	15	0(0%)	0(0%)	1 (6.6%)	0 (0%)	0 (0%)	9 (60%)	4 (26.6%)
Chicken	15	0(0%)	0 (0%)	1(6.6%)	0 (0%)	0 (0%)	7 (46.6%)	2 (13.3%)
Turkey	10	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (20%)	0 (0%)
Total n (%)	40	0(0%)	0(0%)	2(5%)	0 (0%)	0 (0%)	18 (45%)	6 (15%)

Table 5. Prevalence of co-infected *E. coli* pathotypes in tested meat samples

E. coli pathotypes					Source and number of samples			
EHEC	EPEC	ETEC		EAEC	Beef	Chicken	Turkey	Total
(VT^++eae^+)	($bfpA^+$+eae^+)	ST^+	LT^+	$aggR^+$				
+ve	-ve	+ve	-ve	-ve	1	1	0	2
-ve	+ve	+ve	+ve	-ve	1	0	0	1
-ve	-ve	+ve	+ve	+ve	0	1	0	1
+ve	-ve	+ve	-ve	+ve	1	0	0	1
+ve	-ve	-ve	-ve	+ve	1	0	0	1
+ve	+ve	-ve	-ve	-ve	2	0	0	2

+ve: present ; -ve: absent

Prevalence of staphylococcal enterototoxin genes among *S. aureus* isolates in meat samples are shown in Table 3.

Detection of E. coli *and pathotypes*

The results showed that 95% of meat samples were contaminated with *E. coli.* The prevalence of *E. coli* was 100%, 93.3% and 90% in beef, chicken and turkey meat, respectively. The results showed that 89.5% of meat samples contaminated with *E. coli* belonged to *E. coli* pathotypes tested in this study. The total prevalence of uni-infcted samples with EAEC was 5% and with ETEC was 60%. The presence of more than one pathotype was detected in 21% of the tested samples. Prevalence of *E. coli* pathotypes are shown in Tables 4 and 5.

Discussion

Molecular approaches especially PCR-based technique is considered as a sensitive detection method for specific pathogens. Multiplex PCR assay seems tobe a useful technique for rapid and specific detection of pathogens in food and has been used for the control and prevention of foodborne epidemics (Kawasaki *et al.*, 2009). The findings of the current study showed heavy bacteriological load in different meat types with a total viable count ranging from 4.3 $\log_{10}$ to 5.7 $\log_{10}$ cfu/g for frozen meat and 6.95 $\log_{10}$ to 7.78 $\log_{10}$ cfu/g for fresh meat. This heavy load is considered as an indicator for short shelf life of meat. Such heavy load of bacterial contamination of meat and meat products was also reported to ranged from 5.5 $\log_{10}$ CFU/g to 9 $\log_{10}$ CFU/g (Arain *et al.*, 2010; Awny *et al.*, 2010; Abdellah *et al.*, 2013; Anihouvi *et al.*, 2013). The finding of high count of viable mesophilic bacteria in our study is most likely an indication of open-air meat spoilage. Fresh meat that contains 5 $\log_{10}$ CFU/g to 6 $\log_{10}$ CFU/g of background organisms are inherently safer than those that contain less bioload; however, this hypothesis applies only to harmless bacteria (Jay, 1996). Most of foodborne pathogens have a zoonotic origin and have reservoirs in healthy food animals from which they spread to an increasing variety of foods. *Salmonella* and *S. aureus* are the most common and frequent pathogens responsible for food poisoning and food related infections (Costa *et al.*, 2012). According to WHO, 25% of the diarrhea in foodborne illness is caused by food infected with *E. coli* (WHO, 2006).

Enterotoxigenic *S. aureus* is one of the most economically important foodborne pathogen worldwide. Results of this research showed that 30% of meat samples were contaminated with *S. aureus*, and 75% were toxigenic. The prevalence of *S. aureus* in different food products ranged from 12% to 51% (Adwan *et al.*, 2005; Awny *et al.*, 2010; Vázquez-Sánchez *et al.*, 2012; EI-Jakee *et al.*, 2013). The prevalence of enterotoxigenic *S. aureus* in different food products ranged from 5% to 100% (Adwan *et al.*, 2005; Vázquez-Sánchez *et al.*, 2012). The finding of two samples which were sec^+ or see^+ but $FemA^-$ and non mannitol fermenter is most likely to indicate that these samples were contaminated with *Staphylococcus* coagulase-negative. Such finding is in agreement with previous report on association of such genes with coagulase-negative as well as coagulase-positive staphylococci (Podkowik *et al.*,

2013). Detection of toxin genes by PCR allows the determination of potentially enterotoxigenic pathogen irrespective of whether the strain produces the toxin or not. For this reason, PCR may be considered more sensitive than immunological methods that determine staphylococcal enterotoxins production. Staphylococcal enterotoxins A-E are thermostable and also resistant to gastrointestinal proteases such as pepsin, explaining its ability to remain active after ingestion. Therefore, the presence of *S. aureus* in food can be considered a potential health risk (Adwan *et al*., 2005).

Among various foodborne pathogens, *Salmonella* serotypes are the most common bacteria responsible for foodborne gastroenteritis. There are more than 2500 serovars of *Salmonella* and all are considered as pathogenic. *Salmonella* is considered as a zero tolerance organism in foods and should not be present in food, thus the testing of *Salmonella* is mandatory. *Salmonella* is found anywhere in nature, including the digestive tracts of different animals, poultry products, milk products and seafood. Raw chicken meat is known to be the major source for *Salmonella* food poisoning (Chen *et al*., 2008). The prevalence of *Salmonella* in different food products ranged from 2% to 100% (Cohen *et al*. 2007; Aftab *et al*., 2012; Iyer *et al*., 2013; Anihouvi *et al*., 2013; Adeyanju and Ishola, 2014). The findings of the current study showed that 25% of the tested meat samples were contaminated with *Salmonella*. The incidence of *Salmonella* in meat samples is an alarming figure and more attention is required in this respect.

E. coli has been implicated as an agent of diarrheal disease. Diarrheagenic strains of *E. coli* can be divided into five main categories on the basis of distinct epidemiological and clinical features, specific virulence factors, and association with certain serotypes: EAEC, EHEC, EIEC, EPEC, DAEC and ETEC (Nguyen *et al*., 2005; Gómez-Duarte *et al.,* 2009). Our results showed that 95% of meat samples were contaminated with *E. coli,* of which 89.5% were diarrheagenic. The prevalence of *E. coli* in different food ranged from 11% to 100% (Zhao *et al*., 2001; Ukut *et al.* 2010; Abdellah *et al*., 2013; Iyer *et al*., 2013; Adeyanju and Ishola, 2014). High occurrence of *E. coli* and/or diarrheagenic *E. coli* can be explained due to that this pathogen is a part of the normal intestinal flora in most animals. In 2009, Lee *et al.*,reported the occurrence of 39 pathogenic *E. coli* isolates recovered from different meat types . The isolates were categorized into three virulence groups, comprise of ETEC (43.6%), EHEC (35.9%), and EPEC (20.5%) (Lee *et al.* 2009).

Variations in the prevalence of foodborne pathogens from different food samples in different studies could be due in part to several factors including: differences in the reservoir, ecological origin of pathogenic strains, sensitivity of detection methods, detected genes, number of samples, type of sample, time of sampling and storage conditions (Zhao *et al*.2001; Adwan *et al*., 2005). The finding of high level of bacterial contamination as well as the occurrence of virulence factors in food pathogens strongly indicates the need for the implementation of surveillance programs for food products in Palestine

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