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Incidence of Some Food Poisoning Bacteria in Raw Meat Products with Molecular Detection of *Salmonella* in Al Beida City, Libya

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ABSTRACT

In the current study, a total of 100 random samples of raw meat products including; minced meat and beef burger (50 samples of each) were collected from different markets and butcher shops at Al Beida City, Libya. The collected samples were examined bacteriologically for detection of some pathogenic bacteria including; E. coli, Salmonella and Y. enterocolitica. The obtained results revealed that the overall incidence of Enteropathogenic E. coli in the examined samples of minced meat and beef burger was 12 and 24%, respectively with serological identification of serotypes O91:H21, O121:H7, O78, O124 and O44:H18 with various rates. In addition, the overall incidence of Salmonella spp. of minced meat and beef burger was 6 and 4%, respectively with detection of S. Typhimurium, S. Enteritidis and S. Inganda. Moreover, Y. enterocolitica was isolated with an incidence of 12 and 4%, in minced meat and beef burger, respectively. Finally, a molecular study on the obtained isolates of Salmonella was carried out. The recorded results in this study could be traced back to cross contamination occurred during slaughtering and transportation as well as handling during retailing in markets with absence of awareness about different sources of contamination and measures of personal hygiene of sellers. Finally, the public health hazard of the isolated bacteria as well as the recommended measures to lower these microorganisms in meat products and improve the quality of meat products.

1. INTRODUCTION

During the last few decades, a major development in food chain in the form of expansion of supermarkets that provided consumers with a wide range of retailing meat products, so, consumer's interest in meat as a food has been increased and is reflected by the increasing demand for high quality meat products (Stephan et al., 2003). Meat products are gaining popularity as they represent quick easily prepared meat meals with acceptable price which is within the reach of large numbers of families with limited income. Meat contains an abundance of nutrients required for growth of most microorganisms so good manufacturing practices are very important during the procedures of preparation, handling and storage. The presence of *Enterobacteriaceae* in meat products depend upon the meat used for grinding, sanitary conditions, practices during preparation, time and temperature of processing and storage. Also, during cutting and handling, meat surfaces exposed to ambient air provide excellent media for most bacteria (EFSA, 2014). *Escherichia coli* (*E. coli*) are the most important foodborne pathogen causing food poisoning and many other disease conditions amongst food consumers. *E. coli* is a major component of the normal intestinal flora of humans and other mammals. Some *E. coli* strains represent primary pathogens with an enhanced potential to cause disease after acquiring specific virulence attributes. These virulence attributes are normally encoded on genetic elements that can be exchanged between different strains, the presence of theses virulence genes can magnitude the severity of infection caused by these strains (Li et al., 2005).

Salmonella is an important cause of foodborne disease in humans throughout the world and is a significant cause of morbidity, mortality and economic losses. Salmonella problems can occur in all segments of the food chain (Conchello, 2011). According to the European Food Safety Authority (EFSA) report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015, a total of 94,625 confirmed human salmonellosis cases were reported by 28 European Union (EU) member states, resulting in an EU notification rate of 21.2 cases per 100,000 populations. This was a 1.9% increase in the EU notification rate compared with 2014. There was a statistically significant decreasing trend of salmonellosis in the 8-year period between 2008 and 2015 (EFSA, 2016). *Yersinia enterocolitica* is Gram-negative facultative anaerobic non-spore-forming straight rods or coccobacilli that belong to the family *Enterobacteriaceae* (Bottone et al., 2005). *Y. enterocolitica* is widely distributed in the nature and animals; food and environment are routinely contaminated with this organism. Major reservoir of *Y. enterocolitica* is swine. Furthermore, *Y. enterocolitica* has been frequently isolated from poultry and ready-to-eat foods. However, all strains of *Y. Enterocolitica* are not pathogenic to humans but some strains such as biotypes 1B/O:8, 2/O:5,27, 2/O:9, 3/O:3, and 4/O:3 are human pathogens (Fondrevez et al., 2010). Contamination of raw meat products with foodborne bacteria makes the need for a rapid and accurate method for detection is urgent. In the last few years, the development of Polymerase chain reactions (PCR) techniques has offered the possibility of accelerating a great deal of bacterial identification with limited troubles related to sampling preparations, use of specific media, excessive use of chemicals in the traditional methods (Aymerich et al., 2003).

So, the current study was conducted in a trail to determine the incidence of some food poisoning bacteria in raw meat products including; minced meat and beef burger retailed for sale in Al Beida City, Libya beside application of PCR technique for molecular confirmation of *Salmonella*.

2. MATERIALS AND METHODS:

2.1. Collection of samples:

A total of 100 random samples of minced meat and beef burger (50 / each) were collected from different butcher's shops and supermarkets at Al Beida City, Libya. The samples were kept in a separate plastic bag and transferred directly with a minimum of delay to the laboratory in an insulating refrigerated container under complete aseptic condition to avoid any changes in the quality of the sample.

2.2. Preparation of samples for microbiological examinations:

It was performed according to ICMSF, (1996). 25 g of each sample were aseptically transferred into sterile blender flask containing 100 ml of sterile peptone water 1% and homogenized at 14000 rpm for 2.5 minutes.

2.3. Isolation and identification of *E. coli* (Quinn et al., 2011):

About 25 g of each sample was inoculated primarily into tubes of 9 ml MacConkey's broth and then incubated at 37^{o}c for 24 hours. Then subcultured on MacConkey's agar plates and incubated for 24 hours at 37^{o}c, suspected lactose fermented colonies (pink colonies) were picked up and streaked onto Eosin Methylene Blue (EMB) for another 24 hours at 37^{o}c, suspected colonies (greenish blackish colour with metallic luster) were picked up and kept in slope agar for biochemical identification. Films were prepared from suspected colonies and stained with Gram's stain. The suspected *E. coli* isolates appear as pink colored rod shaped Gram negative bacilli. Isolates that were preliminary identified biochemically as *E. coli* were subjected to serological identification according to Kok et al. (1996) by using rapid diagnostic *E. coli* antisera sets (DENKA SEIKEN Co., Japan) for diagnosis of the Enteropathogenic types.

2.4. Determination of the Salmonella incidence was performed in accordance with ISO standard, (2008).

Samples were suspended with 250 ml of buffered peptone water, homogenized for 30 seconds, and after that were incubated at 37°C for 24 h. After pre-enrichment and incubation, 0.1 ml of the slurry was transferred into 10 ml Rappaport-Vassiliadis medium (bioMérieux, France), which was then incubated at 42°C, for a period of 24-48 h. After incubation, enrichment cultures were seeded onto differential Rambach and XLD agars, which were incubated overnight at 37°C. Colonies with typical growth and clearly differentiated were transferred into cryogenic vials for further testing. Isolates proved biochemically to be *Salmonellae* were subjected to serological identification according to Kauffman white scheme (Kauffman, 1974). Isolates were subcultured on nutrient slope for 24 hours at 37° C. For application of slide agglutination technique, two homogenous suspensions were made on a slide by suspending a

piece of suspected colony in a drop of sterile physiological saline. A drop of each of separate o and H *Salmonella* factors were added separately to each of the suspensions with standard loop thoroughly mixed to bring the microorganisms in close contact with antisera. Positive agglutination occurred within a minute and could be easily seen with the naked eye. A delayed or partial agglutination was considered as negative or false result.

2.5. Isolation of *Y. enterocolitica* (Bercovier and Mollar, 1984):

Another 25 g of each sample were taken under aseptic conditions to sterile homogenizer flask containing 45 ml of sterile peptone water (0.1%). The contents were homogenized at 14000 rpm for 2.5 minutes. The mixture was allowed to stand for 10 minutes at room temperature then 1ml of supernatant was added to 5 ml of trypticase soya broth (TSB) (enrichment broth) and incubated at 25 °C for 24 hours. A loopful of enrichment was transferred to 0.1 ml of KOH (0.5%) in 0.5% saline for 2-3 seconds to suppress background flora after enrichment, then a loopful of enrichment was streaked to MacConkey's plates and incubated at 30°C for 1-2 days. The lactose negatives colonies on MacConkey's agar plates were selected and streaked onto CIN (Cefsulodin, Irgasan, Novobiocin) Agar plates then were incubated at 30°C for 1-2 days. One to five susceptible colonies of typical "bull's eye" appearance (small and smooth, with a red center and translucent rim) on the CIN agar plates were individually isolated and subculture on nutrient agar for biochemical identification by catalase, oxidase, urease production, Simmon´s citrate, the behavior in TSI agar and sugar fermentation tests.

2.4. Molecular identification of *Salmonellae* by PCR:

Detection of InvA gene of the obtained isolates from raw meat products was carried out. *Salmonella* specific primers, S139 and S141 have respectively the following nucleotide sequence based on the invA gene of *Salmonellae*. Oligonucleotide primers were prepared according to Rahn et al., (1992).

Primer	Sequence (5'- 3')	Target gene	Amplicon fragment (bp)
invA	S139: 5´GTGAAATTATCGCCACGTTCGGGCA -3´	invA gene	F284
	S141: 5´TCATCG CAC CGT CAAAGG AAC C -3´		

DNA extraction and molecular identification of *Salmonella* spp. by PCR

Salmonella strains were cultured onto Luria Bartani (LB) broth for 24 hours at 37º C then extraction of DNA was done according to Sambrook et al. (1989). Amplification process was performed according to Singer et al. (2006). The PCR system was programmed for denaturation at 94º C, 30 cycles of denaturation at 94º C for 30 seconds. Annealing at 56º C for 30 seconds and extension at 72º C for 2 minutes was performed. Then the reaction was held at 72º C for 7 minutes, and stored at 4º C. PCR products were electrophoresed at 2% (wt/vol) agarose and 0.5 µg of ethidium bromide (Sigma- Aldrich). The samples were electrophoresed at 85 volt for 20 30 minutes. A 300 nm transillumination was used to detect the bands which were then photographed according to Sambrook et al. (1989).

3. RESULTS

Table (1): Incidence of Enteropathogenic *E. coli* in some raw meat products.

Products / *E. coli* serotypes	Minced meat (n=50)		Burger (n=50)	
	No	%	No	%
$O_{91}:H_{21}$	2	4.0	4	8.0
$O_{121}:H7$	3	6.0	2	4.0
O_{78}	2	4.0	0	0.0
O_{124}	2	4.0	3	6.0
$O_{44}:H_{18}$	2	4.0	3	6.0
Total	11	22.0	12	24.0

Table (2): Incidence of *Salmonella spp.* in some raw meat products

Products / *Salmonella spp.*	Minced meat (n=50)		Beef burger (n=50)	
	No.	%	No.	%
Typhimurium	1	2.0	1	2.0
Enteritidis	1	2.0	1	2.0
Inganda	1	2.0	0	0.0
Total	3	6.0	2	4.0

Table (3): Incidence of *Y. enterocolitica* in raw meat products (n=50)

Raw meat products	Positive	
	No.	%
Minced meat	6	12.0
Beef burger	2	4.0
Total	8	16.0

Molecular detection of *Salmonella* isolated from raw meat products:

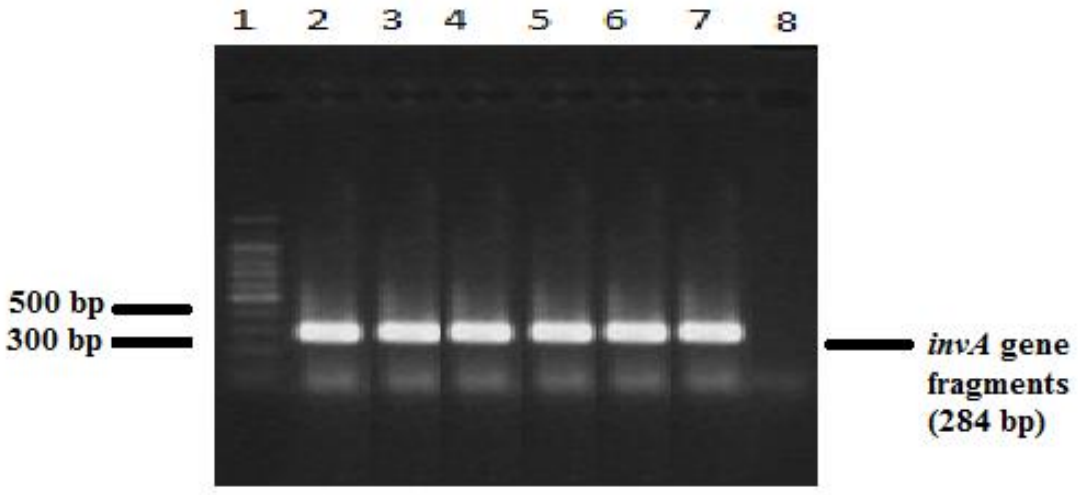

Photo (1): PCR assay showed the predicted amplified product (a 284-bp DNA fragment) from *invA gene* specific for *Salmonella spp.* **Lane (1):** 100 bp molecular weight markers.. **Lane (2):** Positive control. **Lane (3-7):** Positive samples for *Salmonella spp*. (5 isolates). **Lane (8):** Negative control.

4. DISCUSSION

Bacteria multiply rapidly in the presence of moisture, nutrient and specific temperature for growth. Typically bacteria, multiply in temperatures between 5°C and 63°C. Bacterial food poisoning is caused by consumption of food that has been contaminated by bacteria or bacterial toxins as salmonellosis. Poor sanitation, unhygienic food handling and improper preservation and packaging of food consider common cause for food poisoning. Symptoms of food poisoning can range from abdominal pains, vomiting, nausea and fever to hepatic, or renal syndromes, and neurologic especially for toxigenic micro-organisms and their toxin (Zewar, 2008). The data presented in Table (1) showed that the incidence of Enteropathogenic *E. coli* in the examined samples of minced meat and beef burger was 22 and 24%, respectively. Higher results recorded by Hiko et al., (2008), Ibrahim et al., (2012) and Jacob et al., (2013) while they were lower than those recorded by Dambrosio et al (2007), Lindquist and Lindblad, (2009), Mellmann et al., (2011) and Oteiza, (2012). There are two types of *Enteropathogenic E. coli (EPEC):* typical, which possess the EPEC adherence factor (EAF) plasmid and Atypical, which do not possess the EAF plasmid. Currently, the EPEC isolated in industrialized countries are atypical while those from developing countries are typical (Cheasty, 2008). Also, the recorded data in Table (1) illustrated the serotyping of the obtained isolates of Enteropathogenic *E. coli* from

minced meat and revealed the detection of serotypes O_{91}:H_{21}, O_{121}:$_{H7}$, O_{78}, O_{124} and O_{44}:H_{18} with a prevalence of 4, 6, 4, 4, and 4%, respectively while those identified from beef burger were O_{91}:H_{21}, O_{121}:$_{H7}$, O_{124} and O_{44}:H_{18} with a prevalence of 8, 4, 6 and 6%, respectively

The presence of *E. coli* in the examined raw meat products may be attributed to the food stands are simple structures where running water, toilets and washing facilities are seldom available. The washing of hands, utensils and dishes are often done in bowls or pots of water. Also the disinfection is seldom carried out and pests may be attracted to vending sites if there is inadequate sewage disposal. Furthermore, foods prepared at these sites pose health risks as they are ineffectively refrigerated and hygiene principles are not applied properly. Also may be due to handlers contaminate food via manual contact or via the respiratory tract by coughing and sneezing and contamination occurs also after heat treatment of the food (EFSA, 2014). In order to prevent contamination of raw meat products with enteropathogens, the workers must be educated about the sources of *E. coli* to prevent cross contamination and to avoid contamination of such meat meals by fecal matter, also these people must be educated about the principle of personnel hygiene as their hands must be washed after using the toilet, also the water used for washing utensils must be clean and bacteriologically examined. It is forbidden to preserve raw materials with other types of foods as fish, vegetables and fruits to avoid contamination by enteric pathogens which may found in these types of foods. Salmonella was one of the most frequent causes of food borne illness worldwide and transmission involves foods of animal origin (Khaitsa et al., 2007).

The incidence of *Salmonella* in minced meat and beef burger was 6 and 4 %, respectively (Table, 2). The obtained results recorded in examined samples were higher than that recorded by Molla et al., (2006) and they were nearly similar results recorded by Garedew (2015). Detection of *Salmonella* was unsatisfactory and indicated high microbiological risk, *Salmonella* must be absent in relevant products when placed on the market, during their shelf-life (European Commission, 2005). The serotyping of *Salmonella* isolated from minced meat revealed the detection of *S. Typhimurium*, *S. Enteritidis* and *S. Inganda* (2% per each) while *S. Typhimurium* and *S. Enteritidis* were recovered from beef burger. Detection of *Salmonella* in different raw meat products was considered a high microbiological risk, potentially injurious to health and/ or unfit for human consumption (European Commission, 2002). No detection of *Salmonella* in food was the only satisfactory result (EFSA, 2014). The presence of *Salmonella* in the examined raw meat products may be attributed to the bad hygienic practices by the vendors which allow bacteria to come into contact with food and cause food poisoning. Eating food contaminated with Salmonella could cause Salmonellosis, which was one of the most common bacterial food borne illnesses. *Salmonella* infections could be life-threatening, especially to those with weak immune systems, such as infants, the elderly and persons with HIV infection or undergoing chemotherapy. The most common symptoms of salmonellosis were diarrhea, abdominal cramps and fever within 8 to 72 hours (FSIS, 2008).

Yersinia enterocolitica as a member of *Enterobacteriaceae* family is a Gram-negative non-spore-forming rod. It is a psychrotrophic bacterium and able to survive and multiply at refrigerator temperature (Annamalai and Venkitanarayanan, 2005). *Y. enterocolitica* is an enteric pathogen which commonly causes acute enteritis associated with fever, bloody diarrhea, and inflammation of lymph nodes which frequently leads to unnecessary laparotomy due to pseudoappendicitis in humans (Vlachaki et al., 2007). Although contaminated food is the main source of human infection due to *Y. enterocolitica,* animal reservoir and contaminated environment are also considered as other possible infection sources for human in epidemiological studies (Rahman et al., 2011).

The recorded data in Table (3) illustrated that the incidence of *Y. enterocolitica* in the examined samples of minced meat and beef burger was 12, and 4%, respectively.

This result was lower than that recorded by Ahmed and Mohamed, (1998) who could isolate *Y. enterocolitica* with an incidence of 7.5% and Mousa et al., (2014) who found that incidence of identified *Y. enterocolitica* isolated from beef burger was 16%. The prevalence of gastrointestinal illness due to *Y. enterocolitica* was investigated successfully in developing countries like Iraq (Kanan and Z. A. Abdulla, 2009), Iran (Soltan-Dallal and Moezardalan, 2004) and Nigeria (Okwori et al., 2009) and highlighted a major underlying food safety problems in low- and middle-income countries.

Concerning the use of PCR as sensitive and specific diagnostic tool for the detection of *Salmonella* species in raw meat products, 5 isolates of *Salmonella* were

tested successfully using the 284 bp amplified product from *invA gene* specific for *Salmonella*. Salehi et al. (2007) concluded that the PCR results showed that detection of *Salmonellae* at the genus level and specific primers can potentially permit to more readily evaluate samples for the presence of this organism. Also, PCR assays need 24 hours for results obtaining whereas it needs from 5-7 days to identify *Salmonella* by culture methods.

5. CONCLUSION.

In order to prevent contamination of raw meat with pathogens, the workers in slaughterhouse and butchers in meat shops must be educated about the sources of bacteria to prevent cross contamination from the hide of slaughtered animals during skinning and also to be care during evisceration to avoid contamination of such meat and offal by fecal matter, also these people must be educated about the principle of personnel hygiene as their hands must be washed after using the toilet, also the water used for washing such carcasses and offal must be bacteriologically examined.

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