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The Microorganisms associated domestic and imported meats and the possibility of controlling on it.

Basaad Abedzaid
Dr.Sami Abd AL-Reda
Correspondence should be sent to:Basaad Abedzaid
E.mail: basaad.ikab@uokufa.edu.iq

Summary

This study was designed to isolate and diagnose the microorganisms associated with imported and domestic meat depending on morphological , microscopically , biochemical and physiological properties as well as PCR and vitek techniques. Toxic effect of metabolic compounds was produced by fungal isolate on physiological and biochemical parameters in albino rat males which were also determined in addition to the primary diagnosis for these compounds .In addition to that ,the evaluation of the effect of some natural and chemical compounds on occurrence of these microorganisms in meat samples were investigated.

The results showed that a total number of microorganisms in samples of meat reached $222.71x10^5$ cfc/g in imported meat and 128.66 x 10^5 cfc /g in domestic meat . Five bacterial isolates were isolated from meat samples were belong to *Staphylococcus* , *Escherichia* , *Enterococcus* , *Salmonella* and *Serratia* genera. *Escherichia coli* was high frequency and visible get to 89% and 28.94 % respectively ,while the frequency and visible of *Staphylococcus aureus* were 26.31 % and 70.3 % respectively , as well as the multiplex PCR results confirmed that 6 isolates genome belong to *E.coli* were containing gene *STX* which is responsible for toxin synthesis .

Introduction

Meat plays an important role inthe spread ofinfections, especially if theyare equipped withthe treatmentand manuallywaysunhealthy[1]. As exposedmeatfor contamination andit replacesfrom the fields ofeducationand may continuein the massacresduring theslaughter ofanimals andtreateduntil the arrival ofthis meatto the consumer, making it and its products of inferior qualityorunfit forhuman consumption[2].Meat can be exposed tocontamination oflarge numbers ofmicroorganismsduringthe slaughterand other transactionsmay reachmeattypes and numbersofmicroorganismsunwanted andwhich have an effect in corruption ofthis meatormakingmodefor the transfer ofmicroorganismspathogenic tothe consumer.Due tothe multiplicity ofsources of contaminationand the presence ofa suitable environmentfor the growth andmultiplication ofgerms, it is expected to have all kinds ofbacteriaonthe surface of themeat,but theconditions of storage of meatare suitable forthe growth of psychrophilic bacteria andshatteredthe proteinisthe most important . The species of bacteria foundinmeatare*Proteus, Pseudomonas, E.coli, Staphylococus aureus, Salmonella*, [3].

As a result ofthe economic opennesshappening inIraqand the absence ofwatchdogs, companies began toimportmany kinds offrozen meat from different origins without considering the guidelines for meat quality standardswherethemeatmay be contaminatedwith bacteriaand fungi . In order provide an information on microbial contamination thatcould be happenedto themeatof domestic and imported to Najaf province markets .

Materials and Methods

Preparation of solutions , reagents, dyes and media used in the study according to Collee *et al* .,(1996).

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Isolation and Diagnosis of Bacteria -Samples Collection:

One hundred eighty samples of chicken meat , beef and fish (imported and domestic) in the markets of the city of Al-Najaf by a sixty samples of chicken, sixty samples of beef and sixty samples of fish, 5 g were taken from each sample and placed in 5 ml of broth media in test tube and transported to the laboratory in portable container, then incubated for 18-24 hrs at 37C˚, this study take place during the period from December 2011 to June 2012.

Diagnostic of Bacteria - Phenotype and microscope characteristics

Diagnosis to all isolated bacteria was based on morphological characteristics of colonies, Gram stain, in addition to biochemical tests.[4]

Biochemical tests- Depending on the methods in [5]

Definitive Identification via VITEK 2 – Compact .

Were done according to the manufacturer's instructions (Biomerieux) as follow

1. Three ml of normal saline was placed in plane test tube and inoculated with a lope full of isolated colony.
2. The test tube was inserted into a Dens Check machine for standardization of colony to McFarland's standard solution ($1.5x10^8$ cell / ml)
3. The standardized inoculums were placed into the cassette and a sample identification number entered into the computer software via barcode.
4. The VITEK 2 card type was then read from barcode placed on the card during manufacture and the card was thus connected to the sample ID.
5. The cassette was placed in the filler module, when the cards were filled, transferred the cassette to the reader/ incubator module.
6. All subsequent steps were handled by the instrument, the instrument controls the incubation temperature, the optical reading of the cards and continually monitors and transfers test data to the computer for analysis. When the test cycle is completed, the system automatically ejected the cards into a waste container.

Detection of *STX* gene in genomic of *E.coli* isolates by polymerase chain reaction (PCR) technique .

The PCR assay was used for detection of *STX* gene in *E.coli* genomic isolates and done according to following steps :

Genomic DNA extraction. Genomic DNA of *Escherichia coli*. isolates were extracted by using Genomic DNA Kit, as following steps: 1- 1ml of incubated cultured bacterial cells was transferred to a 1.5 ml microcentrifuge tube.

2- Then centrifuged in high speed centrifuge at 15000 rpm for 1 minute then the supernatant discarded.

3- Lysozyme buffer(300 µl) were added to the tube and re-suspended the cell pellet by shaking vigorously by vortex, .

4- Then incubated at 60 c for 10 minutes ,until the sample lysate and the tubes inverted every 3 minutes through incubation periods.

5- Protein Removal Buffer (100µl) were added to each sample lysate and mixed by shaking vigorously for 10 seconds.

6- Then the tubes were incubated at ice 5 minutes and then centrifuge at 15000*g for 3 minutes .

7- Transfer the supernatant to a clean 1.5 ml microcentrefuge tube.

8- Add 300 ml of Isopropanol and mix by inversion and centrifuge at 15000 *g for 5 minutes .

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8 Discard the supernatant and add 300ml of 70 % ethanol to wash the pellet and then centrifuge at 15000*g for 3 minutes

9- Discard the supernatant and air –dry the pellet for 10 minutes . 10- Add 50-100 ml of TE Buffer and incubate at 60C° for 30-60 minutes to dissolve the DNA pellet .During incubation ,tap the bottom of the tube to promote DNA rehydration.

Genomic DNA Profile :

The extracted genomic DNA from Bacterial or fungal samples was checked by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry Chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 1µl of ddH2O onto the surface of the lower measurement pedestal.
3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of the appropriate.
4. Blanking solution was added as black solution which is same elution buffer of RNA samples.
5. After that, the pedestals are cleaned and pipet 1µl of DNA sample for measurement.

Preparation of PCR master mix for (stx). PCR master mix reaction was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions as showing in Table:(1).

Table (1): component of PCR master mix reaction

PCR Master mix reactionComponents		Volume
PCR PreMix* (Lyophilized)		5ul
DNA template		5ul
Primers	F. primer	1.5ul
	R. primer	1.5ul
PCR water		7ul
Total volume		20ul

PCR Thermocycler Conditions: PCR thermocycler conditions were done by using convential PCR thermocycler as following table(2):

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Table (2) :Program of PCR technique.

PCR cycle	Repeat	Temp.	Time
Initial denaturation	1	95C°	5min
Denaturation	30	95C°	5sec.
Annealing		55C°	30sec
Extension		72C°	45sec
Final extension	1	72C°	7min
Hold	-	4C°	Forever

Gel electrophoresis

PCR products of stx gene was analyzed by loading in 1.5% agarose as following steps:
1- 1.5% agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 C° for 15 minutes, after that, left to cool 50 C°.
2- Then 3µg of ethidium bromide stain were added into agarose gel solution. 3- agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of 100bp Ladder in one well.
4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 80 volt and 60 AM for 1.5 hour. 5- PCR products 231 bp as specific for stx gene were visualized by using gel documentary system.

Evalution efficiency of some controlling programs to prevent contamination of meats.
1-Prepration of meat samples Collection 20 samples of domestic meats (cow meats)from local markets of Najaf , each sample weight was 1 kg ,so the total weight was 20 kg then all the meat samples are mixed and taking 12 kg from the total weight and cut into small pieces
2- Treatment of meat, meat (12 kg) divided into nine parts and treatment as the following:

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No.	Treatments	Description of treatments
1	Vinegar	The meat dipping (1 kg)in vinegar for 5 minutes
2	Sodium Hypochloride	The meat dipping (1 kg)in Sodium Hypochloride for 5 minutes
3	Heat	The meat (1 kg) treatment with heating water (100 C°) for 10 minutes
4	Freezing	The meat was storage in deepfreeze (-20 C°)
5	Antibiotic	The meat (1 kg)treatment with 100 mg pencillin per 1 kg meat
6	Vinegar+ Heat	The meat (1 kg) treatment first with heating (100 C °) for 10 minutes ,then treated with vinegar
7	Heat+Sodium Hypochloride	The meat (1 kg) treated first with heating (100 C°) for 10 minutes ,then treated with Sodium Hypochloride
8	Heat +Antibiotic	The meat (1kg) treated first with heating (100C°) for 10 minutes ,then treated with 100 mg pencillin per 1 kg meat
9	Vingar +SodiumHypochloride	The meat dipping (1 kg)in vinegar for 5 minutes,then treated with Sodium Hypochloride
10	Vingar+Antibiotic	The meat dipping (1 kg)in vinegar for 5 minutes,then treated with 100 mg pencillin per 1 kg meat
11	Sodium Hypochloride+Antibiotic	The meat dipping (1 kg)in Sodium Hypochloride for 5 minutes,then treated with 100 mg pencillin per 1 kg meat
12	Control	The meat without treatment

Then putting each treatment of meat in pot and storage under the laboratory condition to 24,48 and 72 hours .

3- Calculate the total number of microorganisms . Taking 10 gram from each treatment and putting in electric blender 10 ml D.W. and mixed for 10 minutes , after that series dilution to work until 10^{-2} in all of the treatment ,except for the control treatment was 10^{-5} then add one ml from this mixture in plate (petri-dish) containing Nutrient agar media ,this process replicated three times (3 replication) . The plates incubated under 37C° for 24 hour after the time and calculated the total number of bacteria . Also the total number of microorganisms account after 48 and 72 hours from the storage of the meats .

Statistical analysis .

All experiments carried out in accordance with the random entire design C.R.D (Complete Random Design) as experiments the mono-and bi factor, and averages were compared according to least significant difference method L.S.D (Less Signification Differences). Below the level of probability (0.05[6]

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Result and Discussion

Isolation and diagnose of microorganisms associated with meat . Bacteria isolated from meat (domestic and imported) .

The results showed that all the samples that have been collected gave appositive result to isolates the bacteria . In the domestic meat as (beef, chicken and fish) they were amended by the total number of microorganism ($218.33X10^5$,$204X10^5$,$241X10^5$) cfu /g of meat samples respectively . In samples of imported meat(beef, chicken, fish) the over all rate of microorganism numbers is ($227.67x10^5$,$229.67x10^5$,$215.67x10^5$) cfu / g respectively of meat sample (Table 3).

Through the production of bacteria note that the number of bacteria that have been isolated from meat domestic types were large numbers compared with meat imported because of the availability air temperature ranging between (33-38)C° in that period of the year, suitable for the growth of bacteria. As for meat imported We note that the samples meat contained high numbers of bacteria, which indicates a lack of adoption of circumstances storage healthy while imported or traded until they reach the consumer as well as the large number of melt, as it contributes to the process of dissolving in providing conditions conducive to the growth of these microorganism sand then deterioration of the quality of the meat and damage during the period of storage [7].

Table (3): Total number of microorganism in one gram of meat .

Type of Meat	Total number of sample(180)		Total number cfu /g	
	Domestic meat	Imported meat	Domestic meat	Imported meat
Beef	30	30	$21.8 x10^6$	$22.7 x10^6$
Chicken	30	30	$20.4 x10^6$	$22.9 x10^6$
Fish	30	30	$24.1 x10^6$	$21.5 x10^6$

While These results indicatea lack of conditions of storage of the meat so that the stored meat must be done under conditions of freezing(-18) C° and that this class will stop the growth of microorganisms and determine prepared. May also attributed the cause of the differences occurring in the current study to the influence of some external influences such as location, transportation, heat, manual handling unhealthy as well as ways of developing and preserving of meat inside butcher shop sand massacre[8].

[9] showed the bacterial aerobic ranged between ($3x 10^4$ - $1.72 x 10^6$) cell bacterial / cm^2 of the samples of meat in the butchers shops in Basra, Alfattli (2010), recorded that highest rates of growth of bacteria was in the domestic meat and this was confirmed by our study, current hitting ($222.72x10^5$) . A study carried out by Dempster (1986) noted that the samples of frozen meat containing the rate of bacteria between 10^3- 10^7 cell bacteria / g meat in a study conducted for detecting microbial contaminants in chicken meat frozen explained AL-Obaidi (1995) contamination of chicken meat frozen some bacterial species. Note that there is little difference in the percentage of contamination between meat of domestic and imported where increasing proportion of bacterial contamination in domestic meat with him in imported and is due to the impact of the freeze in the inhibition of microbial growth in meat imported from the freeze part of the water available for the activity of microbes and thus decrease the value of water activity in a food and this in turn leads to prevent activity microbes . Tai (1987) reached in terms of the lack of available wateris not frozen leads to increase salt in it and in this risk on the microbial cells and bio-effectiveness.

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Also Pearson and Tauber (2002)in a study of them, to inhibite microbial activity when the temperature is less than a degree thermal minimum for growth and private microorganisms loving temperatures medium (mesophiles). Bacteria differ in their sensitivity to extreme freezing ,possibly due to the nature of theen zymes and its mutations differ among themselves by the types of bacteria that bacteria differ in their resistance to the freezing process and usually Gram positive bacteria would be more resistant to the freezing process of Gram negative bacteria as molds are more resistant to freezing of yeasts[10]

According to central Agency for standardization and Quality control in Iraq ,determined the total number permissible of microbial frozen meat is not more than 10 cell /g .

Diagnosis of bacterial isolates .

Five genera and four species diagnosis according to cultural and morphological properties and by using biochemical tests (Table 4) depending on Baron *et al.*, (1998) and Collee *et al*., (1996). Also the Vitek-2 system was used for confirming identification of bacterial species. The results showed that *E.coli* was highly frequenty and visible reached 28.94 % and 89% respectively .While the frequency and visible percentage of *Staphylococcus aureus* were 26.5% and 70.3% respectively. *Serratia* spp. Reported less frequency and visible get to 10.52% and 23.4% respectively (Table 4).These results are in agreement with Okonko *et al*., (2009) and Alfattli, (2010) who reported that *E.coli* and *S.aureus* were most common contamination of meats .The reason for the spread of these bacteria could be due to possess many of mechanisms as producing some enzymes and heat resistance exotoxins . As well as the cooking meat cause the kill of their contamination bacteria ,without breaking down these toxins .Also characterized by their ability to grow in variable environmental conditions of temperature and pH[11,12] showed the domestic meat and imported meat were contaminated with *E.coli* ,*S.aureus* and *Enterococcus feacalis* especilly in the middle parts the meat.

Table(4):Percentages of the frequency and the visible of bacteria.

Type of bacteria	frequency (%)	Visible (%)
E.coli	28.94	89
S.aureus	26.31	70.3
Enterococcus feacalis	18.42	66.4
Salmonella spp	.7815	54.6
Serratia spp	10.52	23.4

Table(5):Biochemical and other testes of bacteria isolates.

Tests	*Serratia spp*	*Salmonella spp*	*Enterococcus feacalis*	*S.aureus*	*E.coli*
Gram stain	—	—	+	+	—
Motility	+	+	+	–	+
Hydrogen sulfide	—	+	–	–	—
Indole	—	—	—	—	+
Methyl red	+	+	—	—	+
Voges proskauer	+	–	—	+	—
Urea hydrolysis	–	–	–	+	+
Gelatin hydrolysis	+	—	—	–	–
Citrate	+	+	–	+	—
Oxidase	–	—	—	—	–
Catalase	+	+	—	+	+
Coagulase	–	–	–	+	–

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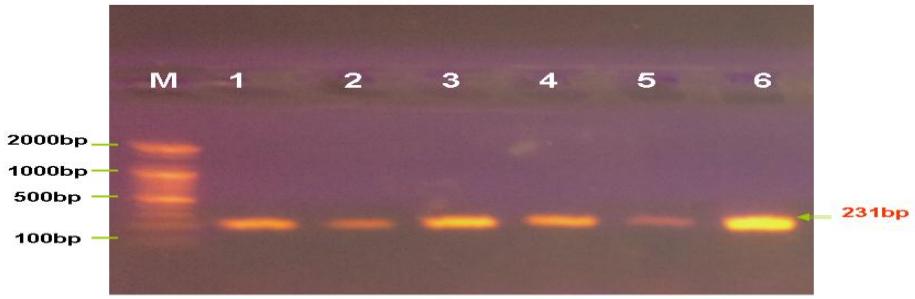
The Vitek-2 system was used for precise and accurate identification of the isolates at generia and species level.

Detection of *STX* gen in *E.coli* isolates by PCR technique .

The results of the polymerase chain reaction (PCR) technique confirmed the DNA of six isolates of *E.coli* bacterium caries for gene *STX* responsible for the encoding of toxin as appeared at the site of molecule DNA standard ladder sized 231 base pairs .(Picture 1).This result agree with findings [13].

Picture (1) : Electrophoresis of product of technique PCR on gel agarose 2%.
M=DNA Leader . 1,2,3,4,5,6=Isolates of *E.coli* which content *(stx)* gene.



Evalution efficiency of some controlling programs to prevent contamination of meats .

The results of this study indicated that the ability of Heat +Antibiotic , Hypochlorite +Antibiotic and Vinegar +Antibiotic treatments were high efficiency to protect the meat from microbial contamination after 72 hours from storage at laboratory temperature .The total number mean of microorganisms were 28.8 ,32.78 and 158.8 cell /gm respectively .Heating treatment decreased the total number of microorganism get 132.2 cell /gm while the total number of microorganism get 217 cell /gm in alone Antibiotic treatment while the total number of microorganisms in frozen treatment get zero. (Table 6). *E. coli* is killed by temperatures of 70°C so food treatment with this temperature should not contain high enough levels of *E. coli* to cause illness in humans. It is very important that every part of the food is heated to at least 70°C. Even properly cooked food should not be stored at room temperature for longer than six hours, as there may still be enough surviving bacteria to establish a population capable of infection[13]

That results agree with Anne and Frederick (1998) who found that the freezing foods infected with *E. coli* stops the growth of the bacterial population for as long as the food remains frozen. At temperatures of 0°C *E. coli* bacteria are unable to divide, keeping the population stable. At temperatures of -18°C *E. coli* begins to die. Storing food at this temperature for a week will reduce the population to about 10 to 30 percent of the original levels. After two weeks at this temperature, survivability drops to about 1%. However, the infection can reassert itself at higher temperatures, and freezing should not be viewed as a method of sterilization.

While the killing of *Enterococcus faecalis, Candida albicans, Staphylococcus epidermidis,* and *E. coli* by NaOCl in concentrations from 2% to 0.03 %[14]

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Table (6): Efficiency of many controlling programs to prevent microbial contamination of meats.

Treatments	Number of microorganism (cfu/g) Time (hour)			Mean of treatments
	24	48	72	
Heat	96.67×10^2	130.00×10^2	170.00×10^2	132.22×10^2
Antibiotic	119.33×10^2	195.00×10^2	336.67×10^2	217.00×10^2
Sodium Hypochlorite	252.33×10^2	386.67×10^2	540.00×10^2	393.00×10^2
Vinegar	159.67×10^2	283.33×10^2	516.67×10^2	319.98×10^2
Vinegar +Antibiotic	56.67×10^2	126.67×10^2	293.33×10^2	158.89×10^2
Heat +Vinegar	163.33×10^2	356.33×10^2	513.33×10^2	344.33×10^2
Vinegar + Sodium Hypochlorite	141.67×10^2	286.67×10^2	536.67×10^2	321.67×10^2
Heat +Sodium Hypochlorite	0.00	15.00×10^2	83.33×10^2	32.78×10^2
Sodium Hypochlorite +Antibiotic	65.00×10^2	185.00×10^2	346.67×10^2	198.89×10^2
Heat +Antibiotic	0.00	20.00×10^2	66.67×10^2	28.89×10^2
Freezing	0	0	0	0
Control(only meat)	159×10^5	380×10^5	522×10^5	353.6×10^5
Mean of time	105.47	198.47	340.33	
L.S.D.(0.05) mean of treatments=15.394 ,mean of time =11.203 ,interference=18.022				

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