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DETECTION OF VIRULENCE GENES IN *ESCHERICHIA COLI* ISOLATED FROM FROZEN MEAT IN BASRAH MARKET

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ABSTRACT

This study was aimed to detect the presence of *Escherichia coli* in frozen meat. A total of 200 samples were collected from Basrah markets in the period extending from September 2015 to March 2016. These samples were composed of 50 frozen fish samples, 50 frozen burger samples, 50 frozen chicken samples and(50) worker's hands swabs. Different techniques were used in this study to evaluate the presence of *Escherichia coli* which contaminate the frozen meat, these techniques included the traditional bacteriological assays, commercial identification kit (API 20 E) and molecular techniques (PCR). Results of these techniques indicated 25 (12.5%) samples were positive to *Escherichia coli* , as identified by API 20 E system. The results of 25 isolates of *Escherichia coli* which confirmed by PCR, These isolates were subjected to PCR [*sta* gene, *stb* gene, *lt* gene and *uspA* gene]. The results PCR confirmed only 16 of these isolates contain *sta* gene and 5 of these isolates contain *uspA* gene ,While isolates do not contain the gene *stb* or *lt* genes.

INTRODUCTION

Raw meat has enriched nutrient composition with water activity ranged from 0.98 to 0.99 and pH ranging from 5.5 to 6.5, and all of these properties support the growth of most contaminating microorganisms [1]. Contamination of raw meat is one of the main sources of foodborne diseases [2]. Unfortunately, the presence of microbs contaminants in meat products cannot be detected visually [3], this increases the risks associated with foodborne microbs and the incidence of human illnesses [4]. The two primary kinds of bacteria which are of consequence are pathogenic bacteria and spoilage bacteria. Spoilage bacteria are generally not harmful, but they will cause food to deteriorate or lose quality by developing a bad odor or texture. Pathogenic bacteria are those such as *Salmonella*, *Escherichia coli* O157:H7, *Campylobacter jejuni*, *Listeria monocytogenes*, and *Staphylococcus aureus*, all which cause

food-borne illness and cannot be seen or smelled [5]. *E. coli* is known to affect the synthesis of vitamin K in the host. Certain *E. coli* strains might also serve as an important factor for inhibition of the growth of enteropathogens [6]. Moreover, *E. coli* is also capable of surviving in the environment, water, and food, and spreading efficiently [7].Thousands of serotypes of *E. coli* species, in the *Escherichia* genus, within the family of *Enterobacteriaceae*, form the intestinal bacterial group described as gram negative, non-sporulating facultative anaerobic rod, usually motile by peritrichous flagella [8]. Pathogenic *E. coli* that do not belong to the normal microbiota, harbor virulence factors, such as adhesions, invasins, entero- and cytotoxins encoded by extrachromosomal plasmids, chromosomal pathogenicity islands, or bacteriophage integrated virulence factors for defeating host defences in order to cause intestinal and extra-intestinal diseases [9].

MATERIALS AND ETHODS

Samples collection:

The samples were collected from different local markets in Basrah city . A total of 200 samples were collected in the period extending from September 2015 to March 2016, including 50 samples of each frozen burger, frozen chicken frozen fish and worker's hands.

Isolation of bacteria :

The samples were collected in a sterile containers and immediately transported inside ice box to the laboratory for bacteriological analysis. The basal medium is trypticase soy broth (TSB) supplemented with (1.5 g) bile salt and dipotassium hydrogen phosphate for each was adjusted at PH 7.4±2 after autoclaved and cooled to 56 °C in water bath,Vancomycin (4 mg/L) were added. The medium was used to enhance the growth of *E. coli* and partially inhibit other bacteria [10]. Tubes were incubated at 37°C for 18 hrs. After enrichment period of samples, a loopfull of bacterial growth from the TSB-V broth was transferred and streaked on the surface of EMB and MacConkey agar then incubated overnight to identify lactose fermentation and metallic sheen appearence . Typical colonies on MacConkey agar and EMB were streaked on the surface of MacConkey sorbitol agar which was composed of 1% sorbitol instead of lactose in standard MacConkey agar, this medium was also supplemented with cefixime 0.05mg/L, potassium tellurite 2.5mg/L to be used as selective medium and incubated for additional overnight to identify non sorbitol fermented *E. coli* (NSFEC).The colonies of EHEC grown on SMA-CT are small, circular and colorless [11].

Identification : All suspected colonies on MacConkey agar and EMB were streaked on the surface of pre-dried nutrient agar plates, in a manner which allowed well isolated colonies to develop. The inculcated plates were incubated at 37C˚ for 24 hrs. Thus, the pure colonies obtained was used for primary identification by biochemical tests such as indole test , triples sugar iron (TSI), citrate test and, oxidase test.

API 20 E

Further confirmations were done by using API 20E test kit (BioMérieux, Inc., France). The plastic strips holding twenty mini-test tubes were inoculated with the saline suspensions of the cultures according to manufacturer's directions. This process also rehydrated the desiccated medium in each tube. A few tubes were completely filled (CIT,VP and GEL), and some tubes were overlaid with mineral oil for anaerobic reactions (ADH, LDC, ODC, H_2S, URE). After incubation in a humid chamber for 18-24 hrs at 37°C, the colour reactions were read (some with the aid of added reagents as supplied by the kit). The data were analyzed by the manufacturer's software [12].

PCR analysis

Extraction of bacterial DNA: This procedure was done by using commercially available DNA extraction and purification kit (Geneaid, Korea).

Preparing the Primers.

Oligonucleotide primers were prepared depending on manufacturer instruction. Sequence of priers were listed in table 1.

The electrophoresis of amplified product was carried out in (1.5)% agarose gel using (7)µl DNA ladder as molecular weight marker and (7)µl of PCR reactions at (70)V for (30) min then at (80)V for (20) min. The gel was visualized by UV transilluminator then photographed.

Table: 1. Oligonucleotide primers for PCR amplification

S	Primer	Sequence (5′→ 3′)	L (bp)	P (bp)	Reference
sta gene	F	GGGTTGGCAATTTTTATTTCTGTA	24	183	Nguyen *et al*.,2009
	R	ATTACAACAAAGTTCACAGCAGTA	24		
stb gene	F	ATGTAAATACCTACAACGGGTGAT	24	360	Nguyen *et al*.,2009
	R	TATTTGGGCGCCAAAGCATGCTCC	24		
lt gene	F	TAGAGACCGGTATTACAGAAATCTGA	26	282	Nguyen *et al*.,2009
	R	TCATCCCGAATTCTGTTATATATGTC	26		
uspA gene	F	CCGATACGCTGCCAATCAGT	20	884	Chen&Griffitha 1998
	R	ACGCAGACCGTAGGCCAGAT	20		

❖ **L:** Length of primers**;** **S:** Specifity**;** **P:** Product size

RESULTS

Isolation and characterization

The growing colonies on Eosen-methylene blue (EMB) agar had green metallic sheen appearance and constituted 25 (12.5%) out of 200 samples. On MacConkey agar, the growing colonies were two types, lactose fermenting and non-lactose fermenter. The isolates of lactose

fermenting produced pink to red colonies. While the non-lactose fermenting, produced colorless colonies. The suspected colonies of EHEC on macConkey sorbitol agar were small, circular and colorless with smoky center (1-2) mm in diameter.

Results of conventional biochemical tests

Specific biochemical tests were used for the detection of lactose fermenting and non-lactose fermenting isolates on MacConkey agar .These biochemical tests were TSI test, citrate utilization, Indole test, oxidase test and urease activity (table 2).

Table (2) Comparison of the isolates according to the conventional biochemical results.

Biochemical tests	Results	No. of results of lactose fermenting	No. of results of non-lactose fermenting
TSI test	Positive	25	47
	negative	0	0
Urease test	Positive	0	18
	negative	25	29
Simmon's citrate	Positive	0	47
	negative	25	0
Oxidase test	Positive	0	47
	negative	25	0

Results of using API 20 E in identification

Using of API 20 E system revealed that25(12.5%) of isolates were identified as *E coli* (table 3).

Table (3) Distribution of *E. coli* according to API 20 E.

Sample	No. of examined samples.	No. of positive	% of Positive
Frozen fish	10	10	100
Frozen burger	7	7	100

Frozen chicken	8	8	100
Worker's hand	0	0	0

Molecular identification

A total of (25) of isolates which were identified by API 20 E were subjected to DNA extraction. PCR assay for the presence of *sta* gene (183bp) (Figure 1) , *uspA* gene (883bp) (Figure 2) ,While *stb* gene (570bp) and *lt* gene (516bp) not present in any isolates.

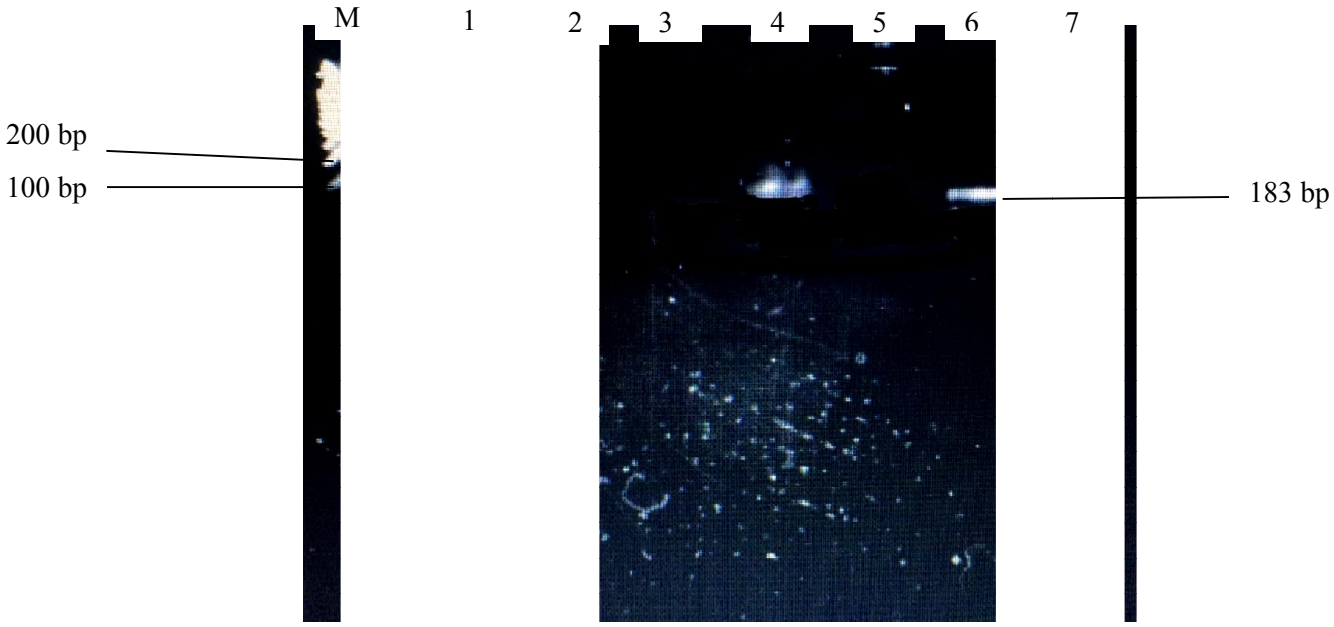

Figure (1): PCR amplification mixture was run on 1.5% agarose gel stained with ethidium bromide. Lanes: M, Marker. 1,4 and 6 positive for *sta* gene (183bp) of *E.coli.*

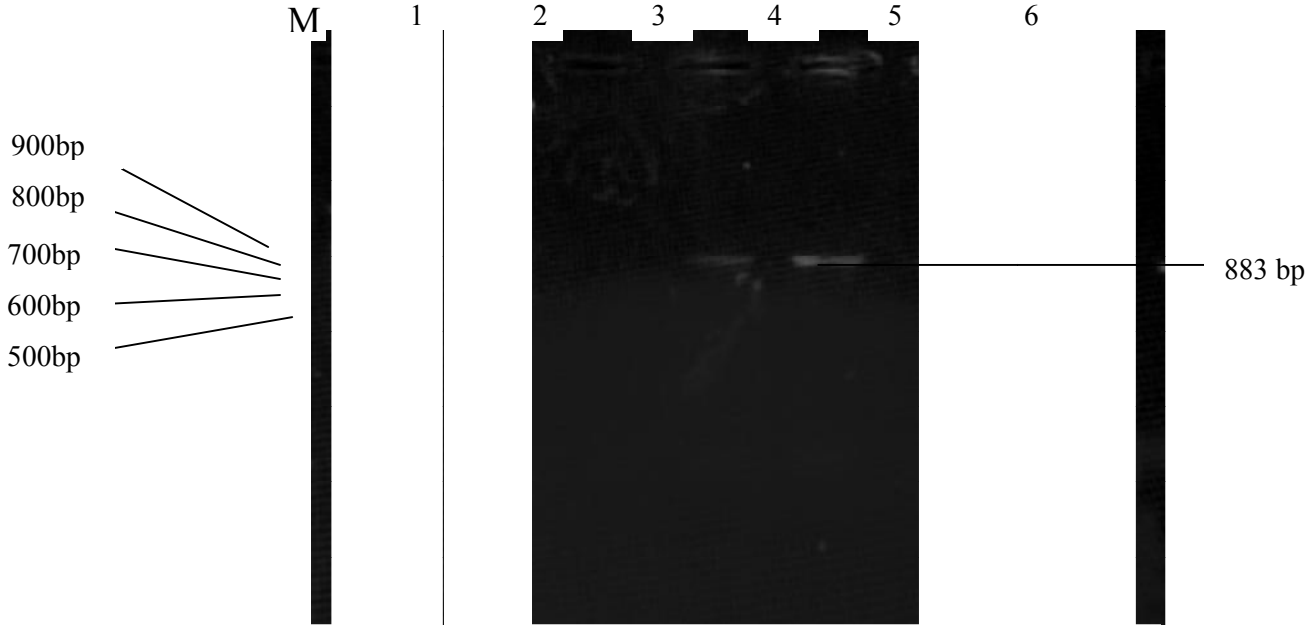

Figure (2): PCR amplification mixture was run on 1 % agarose gel stained with ethidium bromide. Lanes: M, Marker. 3 and 4 positive for *uspA* gene (883bp) as *E.coli.*

Distribution of *E. coli* in tested samples according to PCR results.

According to PCR results out of (25) examined isolates, the positive PCR results were observed in 16 isolates (64%) according to PCR assay using *sta* gene, 5 isolates (20%) according to PCR assay using *uspA* gene , no isolates (0%) according to PCR assay using *stb* gene and no isolates (0%) according to PCR assay using *lt* gene (Table 4) .

Table (4): Molecular identification of *sta* gene from *E. coli* isolated from studied sampled.

Sample	No. of examined samples	*Sta* positive (%)	*UspA* positive (%)	*stb* positive (%)	*Lt* positive (%)
Frozen fish	10	4(40)	1(10)	0(0)	0(0)
Frozen burger	7	7(100)	3(42)	0(0)	0(0)
Frozen chicken	8	5(62.5)	1(12.5)	0(0)	0(0)
Worker's hand	0	0(0)	0(0)	0(0)	0(0)
Total	25	16(64)	5(20)	0(0)	0(0)

DISCUSSION

Food spoilage can be considered as any change, which renders a product unacceptable for human consumption [13].The conventional methods used for *E. coli* detection relies on enrichment the samples in enrichment trypticase soy broth Vancomycin (TSB-V) Medium, followed by culturing on selective media on eosen-methylene blue (EMB) agar, then differentiating on MacConkey agar, MacConkey sorbitol agar and submitting to biochemical

confirmation. Enrichment time allows the target *E. coli* to multiply until reaching a detectable concentration by the conventional and PCR methods. Moreover, after pre enrichment, the number of the dead cells becomes negligible, thus overcoming the problem of the inability of PCR to distinguish between living and dead organisms [14].By culturing the samples on eosen-methylene blue (EMB) agar, the colonies were give a distinctive metallic green sheen and constitute 12.5% (25/200). On EMB agar the form of the colonies were metallic green sheen , therefore, in order to differentiate between them have been cultured on MacConkey agar because this media is differentiated between lactose fermenting and lactose non-fermenting bacteria also lactose fermenting test, it is not part of the API 20 E tests. However, [15]report that, so the colony morphology Lactose fermenter; Flat, dry, pink colonies with a surrounding darker pink area of precipitated bile salts. *E. coli* strain will produce indole from tryptophan; it does not produce hydrogen sulfide, urease, and cannot use citrate as sole carbon source [16].

All these samples were also examined for the presence or absence of *E. coli* O157:H7. plated onto SMAC-CT agar and finally confirmed by PCR [17].The isolates of EHEC O157:H7constitutes 16% (4/25). This is in agreement with [17]who examined 1303 Of the samples for beef burger,43 contained E. coli O157:H7.

After staining with Gram stain , the isolates were submitted to conventional biochemical tests , further, the confirmation of the presumptive isolates was carried out with a commercial bacterial identification kit such as the Analytical Profile Index (API) system. This is in agreement with [18]who noted that, the API-20E is a miniaturized panel of biochemical tests compiled for the identification of groups of closely related bacteria .

The results of conventional biochemical tests had similar to the results of API 20 E. The API 20 E results were revealed 12.5% (25/200) of isolates which were identified as *E. coli*. In this study is in agreement with [19]who isolated *E.coli* from frozen meat in ratio (44%).

The isolation rate of *E.coli* from frozen beef burger samples was (14%)(7/50), this result is in line with [20]who found *E.coli* from this type of samples.

The present of *E. coli* in frozen meat may be constitute a danger for worker and consumers, this point in agreement with [19]who reported that pathogenic *E .coli O157:H7* present potential hazard to human health due to the ability of *E. coli O157:H7* to survive in frozen beef meat.

In this study four methods (conventional, API 20E and molecular) were used to detect *E. coli.* and related genera in raw meat and abattoir environment. The results of this study were

indicated that, the total number of suspected *E. coli* identified by conventional techniques was 25/200 (12.5%), while API 20E system results indicate that all the 25 isolates were identified as *E. coli*, however by molecular identification was 16/200(8%) as *E. coli* by using *sta* gene and 5/200(2.5%) by using *uspA* gene.

Molecular tests have been designed for the detection of many virulence genes and are often the most sensitive methods for detecting them. The primer pairs used in the PCR were designed according to the nucleotide sequences of the four genes. The first primer pair, *sta* -F and *Sta* -R of the *sta* gene [21]will amplify and produce a double-stranded fragment of 183 bp. The second primer pair, *stb* -F and *stb* -R of the *stb* gene[21]will amplify and produce a double-stranded fragment of 360 bp. The third primer pair *lt*-F and *lt*-R, of the *lt* gene [21]will amplify and produce a double-stranded fragment of 282 bp . The fourth primer pair *uspA*-F and *uspA* -R, of the *uspA* gene [22]will amplify and produce a double-stranded fragment of 884 bp .

In this study, the isolation rate of *E .coli* 16/25(64%) , by *sta* gene, this result is in agreement with [23]who found *sta* gene in 40% of *E .coli* isolates.

However in this study, the isolation rate of *E.coli* was 5/25(20%) by *uspA* gene this result is less than [24]who found *uspA* gene in 92% of isolates .while the *stb* gene and *lt* gene were absent in other *E .coli*. this result is in agreement with [25].

الكشف عن جينات الضراوة في جرثومة الاشريشيا القولونية المعزولة من اللحوم المجمدة في أسواق البصرة

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فرع الاحياء المجهرية ،كلية الطب البيطري ،جامعة البصرة ،البصرة ، العراق

تم من خلال هذه الدراسة الكشف عن وجود جرثومة الاشريشيا القولونية في اللحوم المجمدة .تم جمع 200 عينة من اسواق البصرة للفترة من شهر ايلول 2015 لغاية شهر اذار2016 .

العينات التي تم اخذها تكونت من 50 عينة من السمك المجمد،50 عينة من البرغر المجمدة ، 50 عينة من الدجاج المجمد و50 مسحة من ايدي العاملين .

كما تم استخدام تقنيات مختلفة في هذه الدراسة لتقييم وجود الاشريشيا القولونية التي تلوث اللحوم المجمدة ، وهذه التقنيات تشمل الاختبارات البكتريولوجية التقليدية ، عدة (API (20 E التجاري والتقنيات الجزيئية (تفاعل البلمرة المتعدد). تشير نتائج هذه التقنيات إلى وجود 25 عزلة من اصل 200 أي بنسبة 12,5% من الاي كولاي باستخدام اختبارات API 20 E .

خضعت هذه العزلات لتفاعل البلمرة المتعدد (جين *sta* و جين *stb*، وجين *lt* وجين *uspA*). وأكدت نتائج تفاعل البلمرة المتعدد ان فقط 16 عزلة من هذه العزلات تحتوي على جين *sta* و 5 عزلات تحتوي على جين *uspA* بينما لا تحتوي العزلات على جين *stb* ولا تحتوي ايضا على جين*lt* .

REFERENCES

1. **Acuff, G. (2005).** "Chemical decontamination strategies for meat". Cited by **Sofos, J. (2005).** Improving the safety of fresh meat. *Boca Raton, FL: CRC Press*., Pp: 350-363.
2. **Podpecan, B.; Pengov, A. and Vadnjal, S .(2007).** The source of contamination of ground meat for production of meat products with bacteria *Staphylococccus aureus*. Slov. *Vet. Res*., 44: 24-30.
3. **Movassagh, M. H.; Shakoori, M. and Zolfaghari, J. (2010).** The prevalence of *Salmonella spp*. In Bovine carcass at Tabriz slaughterhouse, Iran. *Global Veterinaria*.,5(2):146-149.
4. **Ateba, C. and Mochaiwa, B. (2014).** Use of *invA* gene specific PCR analysis for the detection of virulent *Salmonella* species in beef products in the north west province, South Africa. *Journal of Food and Nutrition Research* ., 2(6): 294-300.
5. **International Commission on Microbiological Specifications for Foods.(1996).** Microorganisms in foods 5. Characteristics of microbial pathogens. ICMSF, International Union of Biological Societies. Blackie Academic & Professional, London, England.
6. **Kruis, W. (2004).** Review article: antibiotics and probiotics in inflammatory bowel disease. *Aliment. Pharmacol. Ther*, 20 (4): 75-78.
7. **Faiella-Tommasino, J. and Reigert, H. (2002).** *E. coli* O157:H7–An Emerging bacterial threat. *Clin. Rev*., 12: 47-53.
8. **Scheutz, F. and Strockbine, NA. (2005).** Genus I. *Escherichia*. In: Brenner, DJ. (Eds.) The Protebacteria Part B The Gamma proteobacteria. Springer Bergey's Manual of Systematic Bacteriology., 2 Part B:607-623**.**
9. **Kaper, J. B.; Nataro, J. P. and Mobley, H. L. T. (2004).** Pathogenic *Escherichia coli*. *Natu. Rev. Microbiol*., 2: 123-140.
10. **LeJeune, JT. ; Besser, TE. ; Rice, DH. and Itancock, DD. (2001).** Methods for the isolation of waterborne *Escherichia coli* O157:H7. *Lett. Appl. Microbiol*. , 32, 316–320.

11. **Vincent, NC. ; Veronica, JU. ; Stella, IS. ; Etinosa , OI. and Anthony, IO. (2010).** Multidrug resistance and plasmid patterns of *Escherichia coli* O157 and other *E. coli* isolated from diarrhoeal stools and surface waters from some selected sources in Zaria, Nigeria*. Int. J. Environ. Res. Pub. Health*., 7:3831-3841 .

12. **Imen, B.; Ridha, M. and Mahjoub, A. (2012).** Laboratory typing methods for diagnostic of *Salmonella* strains, the "old" organism that continued challenges, *Salmonella* a dangerous foodborne pathogen.*Infect Immun*., 57: 3159- 3164.
13. **Hayes, P. R. (1985).** Food Microbiology and Hygiene. *Elsevier, London.,* pp: 80 - 139.
14. **Croci, L.; Delibato, E.; Volpe, G.; De Medici, D. and Palleschi, G. (2004).** Comparison of PCR, Electrochemical Enzyme - Linked Immuno Sorbant Assays and the standard culture method for detection *Salmonella* in meat products . *Appl. Environ. Microbiol*., 70(3): 1393-1396.

15. **Mahon, A.R.; Barnes, M.A.; Senapati, S.; Feder, J.L.; Darling ,J.A. and et al. (2011).** Molecular Detection of Invasive Species in Heterogeneous Mixtures Using a Microfluidic Carbon Nanotube Platform. *PLoS ONE*.,6(2):e17280–1-5.
16. **Mahon, CR. ; Lehman, DC. and Manuselis, G. (2007).** Textbook of diagnostic .3rd Ed., St. Louis: Saunders., Pp: 215-216.

17. **Cagney, C.; Crowley, H.; Duffy, G.; Sheridan, J.J.; O'Brien, S.; Carney, E.; Anderson, W.; McDowell, D.A.; Blair, I.S. and Bishop, R.H.(2004).** Prevalence and numbers of Escherichia coli O157: H7 in minced beef and beef burgers from butcher shops and super-markets in the Republic of Ireland. *Food Microbiol.*, 21:203-212.
18. **Modarressi, S. and Thong, K. (2010**). Isolation and molecular sub typing of *Salmonella enterica* from chicken, beef and street foods in Malaysia. *Scientific Research and Essays*., 5(18): 2713-2720.
19. **Abuelhassan, N.N.; Abdul Mutalib, S.; Muin, N.M.; Tibin, E.M.M.; Doni, F. And Yusoff, W.M.W. (2014).** Antibiotic Resistance Pattern Among *Escherichia Coli* O157:H7 Isolated From Retail Imported Frozen Meat. *Asian Jr. of Microbiol. Biotech. Env. Sc.*, 16 (3) : 739-744.
20. **Stanford, K.; Agopsowicz, C.A. and McAllister, T.A. (2012).** Genetic diversity and antimicrobial resistance among isolates of *Escherichia coli* O157: H7 from feces and

hides of super-shedders and low-shedding pen-mates in two commercial beef feedlots. *BMC. Vet. Res*.,8:178.

21. **Nguyen, T. L.; Truong, H. T. and Nguyen, V. G. (2009).** Determining virulence factors of Escherichia coli strains isolated from diarrhea piglets by using PCR method. *J. Sci. Dev.* ,7(2): 187 - 191.
22. **Chen, J. and Griffiths, M.W. (1998).** PCR differentiation of *Escherichia coli* from other Gramnegative bacteria using primers derived from the nucleotide sequences flanking the gene encoding the universal stress protein. *Letters in Applied Microbiology*, 27: 369-371.
23. **Macedo, N.R.; Menezes, C.P.L.; Lage, A.P.; Ristow, L.E.; Reis, A. and Guedes, R.M.C. (2007).** Detecção de cepas patogênicas pela PCR multiplex e avaliação da sensibilidade. *Arq. Bras. Med. Vet. Zootec.* Belo Horizonte., 59 (5).
24. **Osek, J. (2001).** Multiplex polymerase chain reaction assay for identification of enterotoxigenic Escherichia coli strains. *J. Vet. Diagn. Investig.*, 13**:**308-311.
25. **Fasina,F.O.; Bwala, D.G and Madoroba, E.(2015).** Investigation of multidrug-resistant fatal colisepticaemia in weanling pigs. *Onderstepoort j. vet. res.*,82(1):6.