Isolation, Identification and Characterization of *Escherichia Coli* O157:H7 from Poultry Meat - A Worldwide Public Health Threat!

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Abstract

Background
Food borne and meat borne diseases are considered as a major and most important challenge to the public health. Each year millions of human deaths in the world can be attributed to the *Escherichia coli* contaminated foods. Poultry meat is one of the major sources of food borne illness caused by *E. coli*. Hence this study was conducted to isolate, identify and characterize *Escherichia Coli* O157:H7 from Poultry Meat because of its public health significance, moreover to aware people from the spread of meat borne infections.

Methods
Poultry meat samples (chest piece, leg piece, frozen meat, minced chicken meat) were collected from different retail outlets located at Lahore city and processed for isolation of *Escherichia Coli* on different culture media. The positive isolates for *E. coli* were microbiologically identified on the basis of morphology, and confirmed by different biochemical reactions. Furthermore, all the confirmed isolates through biochemical tests were subjected to polymerase Chain reaction (PCR) analysis for presence of *E. coli* O157:H7.

Results
Results showed that overall by traditional culture methods 29 chicken samples on nutrient agar, 21 on MacConkey agar, 15 on SMAC and 15 on BGA were found positive showing 36.25%, 26.25%, 26.25%, 18.75% percentage positivity. A total of 11 samples by biochemical tests and 6 samples through PCR were found positive indicating the 13.75 % and 7.5 % percentage positivity in chicken meat samples respectively. Thus these results indicated that *E coli* O157:H7 is prevalent in Lahore, Pakistan with highest percentage of *E. coli* O157:H7 in minced chicken meat.

Conclusion
E. coli O157:H7 is prevalent in poultry meat in our country as a great hazard to public health. Polymerase chain reaction is sensitive, specific and efficient way for confirmation of *E. coli*. Hygienic measures are recommended as of core importance to avoid food borne illnesses.

Key words
Poultry Meat, Polymerase chain reaction (PCR), *E. coli* O157:H7, Food safety, Public health

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Introduction

Food borne and meat borne diseases are considered as public health threat for both developing and developed countries.[1,2,3] *E. coli* is gram–ve, bacilli, flagellated, motile, oxidase –ve, facultative anaerobe, categorized under the family Enterobacteriacea and is prevalent in six continents.[4, 5] It is most protean bacteria and in variety of hosts causes a range of illness such as septicemia and diarrhea.[6] The infective dose for food poising is very low and causing high morbidity and mortality among human beings. An estimated 1.8 million deaths of children and 2.1 million deaths of adult occur every year in the whole world due to food borne gastrointestinal illness caused by *E. coli*. Every year in developed countries almost 1/3 of human population is affected by food borne illness caused by *E. coli* and in developing countries about 650 million cases were reported.[7]

E. coli can be classified in to two major kinds on the basis of sorbitol fermentation i.e. sorbitol fermenting (SF) *E. coli* and non sorbitol fermenting (NSF) *E. coli*. The non sorbitol fermenting specie *E. coli O157:H7* is said to be major cause of food poisoning among human. This serotype also causes mild illness, diarrhea, vomiting disorder, nausea, renal and liver failure, nervous disorders, paralysis, arthritis, hemolytic uremic syndrome (HUS), thrombocytopenia purpura (TCP), and hemorrhagic colitis (HC) worldwide.[8-14] The primary reservoirs of *E. coli* O157:H7 are cattle and chickens. The major cause of illness of this bacterium is due to contamination of raw vegetables, meat, swimming pools, water reservoirs, dairy, beef and chicken products. Improperly cooked meat and milk is considered as the source of illness due to *E. coli O157:H7*.[9, 15] Accurate diagnosis of causative agent of an infectious disease at early stage is critical for prevention and control. Several

bacteriological (culture media) and biochemical techniques have been used to isolate *E. coli* O157:H7 from different sources. However now a day's chromogenic media are also available which identify the microorganism on the basis of color.[16] Initial identification is done on the basis of biochemical characterization. The confirmatory techniques for *E. coli*, based on immunological assays, includes enzyme linked immune sorbent assay (ELISA), latex agglutination (LA), reversed passive latex agglutination (RPLA). These methods are considered more reliable, sensitive and less laborious as compared to traditional cultural techniques.[16-17] Molecular methods used for confirmation and serotyping of *E. coli* are pulsed field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR). Amongst all above methods only PCR has been found to be rapid and sensitive method for the confirmation of *E. coli* and is considered as best potential tool for the screening of isolates up to serotype level.[18-20]

Pakistan is one of the major consumer of poultry meat and its products.[40] Keeping in view the public health significance of *E. coli* O157:H7 in poultry meat this study was conducted for the prevalence of the organism in retail poultry meat and to standardize procedure that can specifically isolate *E. coli* 0157:H7 from poultry meat. Hygiene measures necessary to minimize or prevent contaminants from meat borne sources are also recommended.

Material and Methods

Collection of Samples

Samples were collected from poultry sale points of different areas of Lahore, Pakistan under aseptic condition in plastic bags. The samples were immediately taken to the laboratory at Veterinary Research Institute (VRI), Lahore and stored at 4° C and processed within 24 hours. Eighty samples (N=80) were collected with 20 pieces from each part. The samples included chicken raw meat (chest piece: n=20, leg piece: n= 20) frozen meat (n=20), and minced chicken meat from Bar B Q shops (n=20).

Enrichment

Collected samples were enriched in tryptic soy broth (TSB) and incubated for 24 hours. For this 1 gm of triturated sample of meat (in tissue grinder) was mixed in 9 ml of TSB in test tubes.

Isolation of *E. coli*

A loop full of microbes from enriched culture was taken and streaked on Nutrient agar (NA), MacConkey agar (MA) Tryptic soy agar (TSA), Sorbitol MacConkey agar (SMAC) and Brilliant green agar (BGA) in biological safety cabinet. Plates were incubated in incubator at 37° C for 24 hours. After 24 hours the colonies were observed and results were recorded.

Identification of *E. coli*

A thin smear was prepared by putting a loop of distilled water loop full of microbes from isolates colonies on glass slide with tooth pick. To see the morphological characteristics all the prepared slide were stained by Gram Staining method. Stained slides were observed under 100X power objectives and results were observed and noted.

Confirmation of *E. coli*

Biochemical Characterization

The suspected micro organisms were analyzed by biochemical testing. TSI test (triple sugar iron agar slant), Indole test, Methyl red test**,** Vogus Proskaur, Catalase test, Urease test, Simmon citrate utilization test, Oxidase test and Carbohydrate fermentation tests.

PCR (Polymerase chain reaction) Technique

In the study simple PCR technique was used for the confirmation of *E. coli* O157:H7. The suspected colonies of *E. coli* were purified by further streaking out on selective media i.e. Sorbitol MacConkey Agar (SMAC) and Brilliant Green Agar (BGA).

Pure colonies were further confirmed by using polymerase chain reaction (PCR) technique. Primer against *f*liCH7 gene (catalogue # PT0400A, amplification product size approximately 625 bp) and rfb O157 gene (catalogue # PT04001, amplification product size 259 bp) were designed.[21-22] Protocols used for PCR technique is described.[22-23]

Gel Electrophoresis

To load on gel, DNA loading dye was added to the samples.

Table 1. Oligonucleotide sequence of Primer for *f*licH7 and r*f*bO157 gene of E. coli O157:H7 used for PCR

Primer	Sequence ($5^{/}$-$3^{/}$)	Amplicon Size (bp)
r*f*bO157	F:CGGACATCCATGTGATATGG R: TTGCCTATGTACAGCTAATCC	259
*f*licH7	F: GCGCTGTCGAGTTCTATCGAGC R: CAACGGTGACTTTATCGCCATTCC	625

Samples were loaded carefully in wells. DNA ladder mix (10KB) was loaded as standard in one of the well. The gel was run at 120 volts about half an hour. The gel was removed and the bands of DNA were visualized under the UV light in the trans-illuminator. The DNA ladder size used for PCR assay was 100 bp as a size of reference. 259 bp and 625 bp were the expected size for r*f*bO157 and *f*licH7 genes of PCR products.

Data Analysis

Isolated samples were statistically analyzed using "minitab 17" through "Chi square test for association". Ap value of less than 0.05 ($P<0.05$) was considered significant. It means that there

was some sort of association exists between the isolated sources of the chicken meat. On the other hand if p value is greater than 0.05 (p>0.05) it indicated that that there is no significant difference between the isolated sources of the chicken meat. It shows that there is significant association exist between the isolated sources.

Results

In this study a total of 80 samples from local chicken raw meat i.e. leg piece (n=20) and chest piece (n=20), processed frozen meat (n=20), local ground/minced chicken meat (n=20) were tested for the presence of *E. coli* O157:H7.

Isolation of *E. coli*

Nutrient Agar

Small, circular, color less, colonies with entire margins and raised elevation were formed on nutrient agar showing the suspected micro organism. Eight samples from leg piece, 6 from chest piece, 5 from frozen processed meat and 10 samples from local ground chicken meat were found positive thus indicating the 40 %, 30 %, 25% and 50 % positivity respectively. Statistical analysis showed no significant difference between the different isolate sources P= 0.363 (P>0.05) which indicated that no association exists between the isolated source of chicken meat (Figure 1(a), Figure 2).

MacConkey Agar

On MacConkey agar *E. coli* produced small, circular, pink color colonies with raised elevation and entire margins indicating the presence of *E. coli*. Six samples from leg piece, 4 from chest piece, 2 from frozen processed chicken meat and 9 samples from the local ground chicken meat were found positive indicating the 30 %, 20 %, 10 % and 45 % positivity rate respectively. Statistical analysis showed no significant difference between the different isolate sources P=0.075 (P>0.05) which indicated that no association exists between the isolated source of chicken meat (Figure 1(b), Figure 2).

Sorbitol MacConkey Agar

E. coli 0157:H7 is unable to ferment sorbitol so it forms small, circular, transparent colonies, with raised elevation and entire margins on SMAC. A total of 5, 3, 1 and 6 samples were from leg piece, chest piece, processed frozen chicken meat and ground chicken meat for *E. coli* were found positive showing 25 %, 15 %, 5% and 30 % positivity rate respectively. Statistical analysis showed no significant difference between the different isolate sources P= 0.184 (P>0.05) which indicated that no association exists between the isolated source of chicken meat (Figure 1(c), Figure 2).

Brilliant Green Agar

The positive samples were further streaked on BGA and *E. coli* and produced small circular colonies with metallic sheen, raised elevation and entire margins. Five samples from leg piece, 3 samples from chest piece, 1 sample from processed frozen chicken meat, and 6 samples from local ground chicken meat were found positive for *E. coli* showing 25 %, 15 %, 5 % and 30 % positivity rate respectively. Statistical analysis showed no significant difference between the different isolate sources P=0.184 (P>0.05) which indicated that no association exists between the isolated source of chicken meat. Figure 1(d), Figure 2.

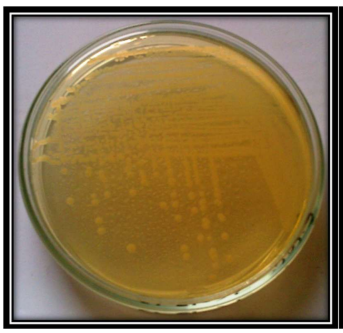

Fig 1(a). *E. coli* colonies on Nutrient Agar

Fig 1(b). *E. coli* colonies on MacConkey Agar

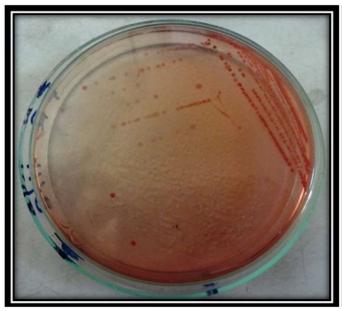

Fig 1(c). *E. coli* colonies on Sorbitol MacConkeyAgar

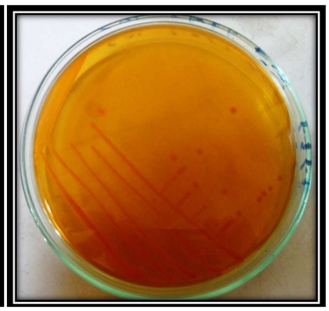

Fig 1(d). *E. coli* colonies on Brilliant Green Agar

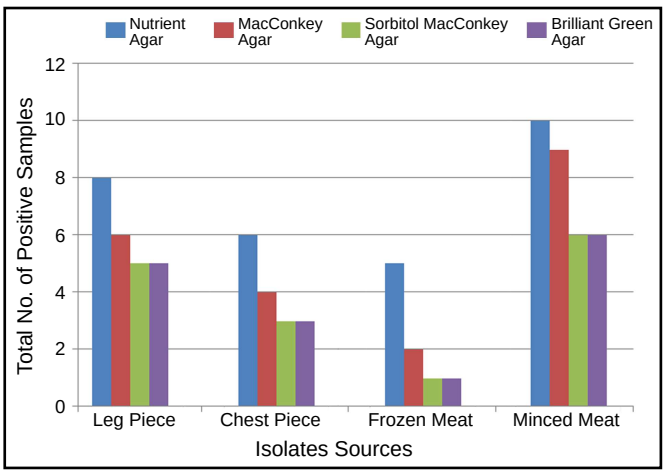

Fig 2. Graphical demonstration of all positive samples of *E. coli* on culture media

Identification of *E. coli*

Microscopic Study

The positive isolated showed pink color, rod shape bacilli in single or pair forms under the microscope 100X power of objective lens (Figure 3).

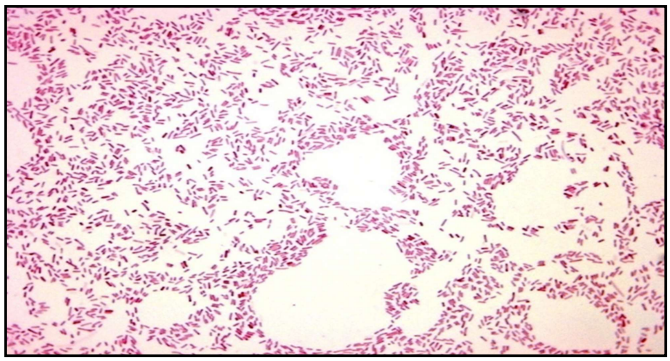

Fig 3. Gram staining (at 100X Power objective)

Biochemical Characterization
Culturally isolated positive samples were further identified by biochemical tests.

TSI Slant Test
On TSI slant yellow butt and yellow slant indicating that the tested microorganism is able to ferment glucose, and lactose which indicated the presence of *E. coli*.

Indole Test
Positive samples were tested for indole test and pink color produced at the junction of tryptone broth and Kova'c reagent, thus confirming the *E. coli*.

Methyl red Test
Samples were tested for methyl red test red color produced at the surface of the broth indicating the positive test for methyl red.

Vogus Prasuker Test
Samples were tested for Vogus Prousker test and no change in color was produced by adding VP reagent to the broth and thus indicating negative test for VP.

Catalase Test
Positive samples showed the bubble formation hence indicating the positive test for *E. coli*.

Urease Test
E. coli is unable to ferment urea thus no color change takes place in urea broth.

Citrate Utilization Test
Samples were tested for Simmon's citrate test and no color change was observed in the tubes indicating negative result.

Oxidase Test
No color produced on filter paper which indicated that the tested organism is oxidase negative.

Carbohydrate Fermentation Test
Culturally positive samples were tested to check the ability to ferment sugars. Positive samples for *E. coli* showed color change due to acid production for mannitol, mannose, lactose, maltose, and arabinose. On the other hand no color change was observed in raffinose, sorbitol and dulcitol indicating positive results for *E. coli.* By biochemical testing 3 , 2, 1 and 5 samples were found positive for *E. coli* from leg piece, chest piece, processed frozen chicken meat and local ground chicken meat (Table 2, Figure 4.), thus indicating the 15%, 10%, 5% and 25% positivity respectively. Statistical analysis showed no significant difference between the different isolate sources P= 0.371 (P>0.05).

Polymerase Chain Reaction (PCR)
The biochemically positive samples were further confirmed by polymerase chain reaction (PCR). Primers for *f*lic H7 gene and rfp O157 were used to isolate *Ecoli* O157:H7. A band of 625 bp of *f*lic H7 gene and 259 bp of rfp O157 gene were detected on agarose gel after amplification. Two samples from leg piece, 1 sample from chest piece and 4 samples from local ground chicken meat were found positive by polymerase chain

Table 2. Results of biochemical test for detection of *E. coli*, through biochemical tests.

Oxidase	-	-	-	-
Citrate Utilization	-	-	-	-
Voges Proskaur	-	-	-	-
Methyl Red	+	+	+	+
Catalase	+	+	+	+
Urease	-	-	-	-
Indole	+	+	+	+
TSI	+	+	+	+
P Value	P =0.371			
% age Positivity	15	10	5	25
Positive Samples	3	2	1	5
No. of Samples	20	20	20	20
Isolate Sources	Leg Piece	Chest Piece	Processed Frozen Chicken Meat	Ground Chicken Meat
	Chicken Raw Meat			

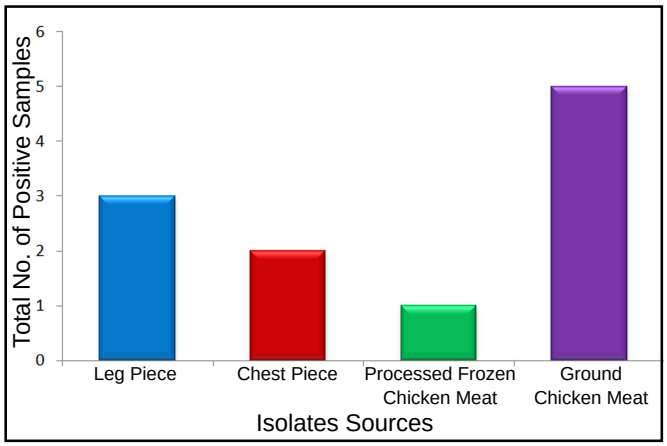

Fig 4. Graphical demonstration of Positive Samples by Biochemical tests

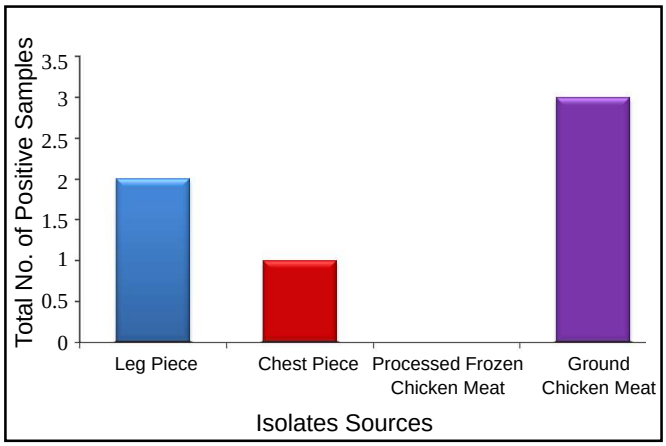

Fig 6. Graphical demonstration of Positive Samples by PCR

reaction and the positivity rate was 10%, 5%and 20% respectively. No sample was found positive from processed frozen chicken meat. Statistical analysis showed no significant difference between the different isolate sourcesP=0.140 (P>0.05).Table 3, Figure 5-6.

Table 3. Results of PCR for detection of E. coli.

Isolate Source		Total No. of Samples	Total No. of Positive Samples	%age	P Value
Raw Chicken Meat	Leg Piece	20	2	10	
	Chest Piece	20	1	5	
Processed Frozen Chicken Meat		20	-	-	P= 0.140
Ground Chicken Meat		20	4	20	
Total		80	7	8.75	

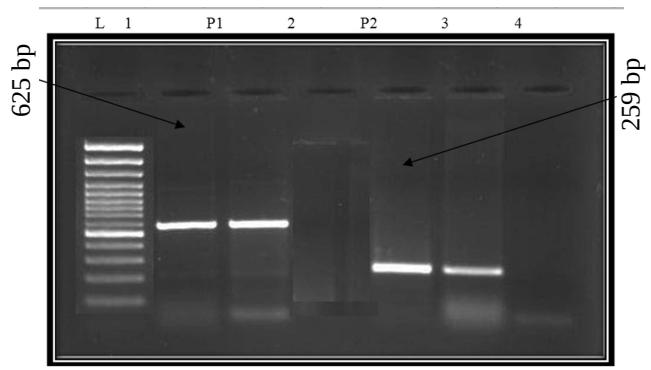

Fig 5. Results of the PCR assay, amplifying 259-bp segment of *rfb*O157 and 625-bp of *flic*H7 gene of *E. coli* O157:H7. Lane L: Ladder sequence of hundred bp, Lane P1 and P2: positive control, Lane 1 and 3: samples.

Over all by traditional culture methods 29 chicken samples on nutrient agar, 21 on MacConkey agar, 15 on SMAC and 15 on BGA on BGA were found positive showing 36.25%, 26.25%, 18.75% percentage positivity. A total of 11 samples by biochemical tests and 6 samples through PCR were found positive indicating the 13.75% and 7.5% percentage positivity in chicken meat samples respectively. Thus these results indicated that *E coli* O157:H7 is prevalent in Lahore. Figure 7.

Discussion

E. coli is frequently occurred in milk, meat and food products. The method of production, transportation, and handling of these products are unhygienic which causes great hazard to public health. All these products are highly enriched source of bacterial contamination, especially in our country with suitable environment. *E. coli* usually is found in normal flora of human and animal intestine and have been identified as leading cause of food borne illness all over the world. *E. coli* and *E. coli* O157:H7 strain has been isolated from meat, milk and vegetables.[24, 25]Therefore this study was conducted to check the prevalence of *E. coli* O157:H7 from different poultry meat sources and moreover to aware people from the spread of disease due to improperly cooked poultry meat in Lahore, Pakistan. In this study conventional culture methods for bacterial isolation were performed and confirmed through biochemical tests and modern, standardize technique polymerase chain reaction.

The results show that the highest percentage of *E. coli* O157:H7 was observed by bacteriological methods in ground chicken meat (30%) confirmed by biochemical test and polymerase chain reaction (25% and 20%) respectively. These findings are in accordance with a report, which showed that ground beef samples has maximum contamination of *E. coli* O157:H7.[25]

Results from PCR analysis for *E. coli* O157:H7 using primer specific for rfbO157 and *f*liCH7 gene is similar to other studies.[22,23, 26, 31, 34] Our results indicate that the chicken may be

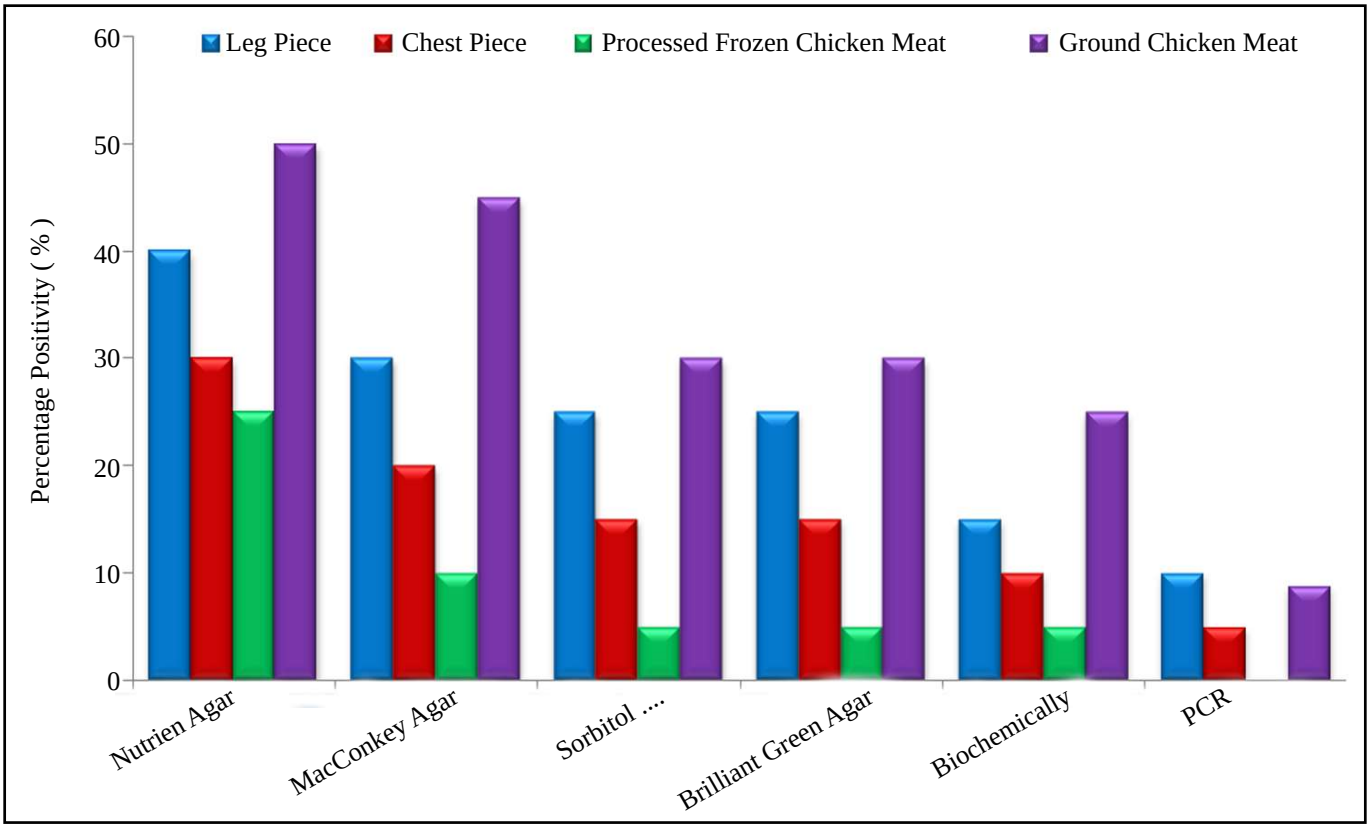

Fig 7. Graphical demonstration of E. coli Positive as a study overview

are servoir of *E. coli* O157: H7inPakistan, as in other countries where beef meat is considered to be reservoir for *E. coli* O157:H7.[22, 35, 36]

PCR results for *E. coli* O157:H7 isolates was higher as compared to few other studies in which the rate of positive isolates through polymerase chain reaction were 4.4%, 2%, 4% and 4.5%respectively.[27, 30, 37, 38] However on the other hand rate of isolation of *E. coli* O157:H7 (8.75%) was lower than that of 16.6% which was observed in another study.[39]

Conclusion

*E. coli O157:H7*is frequently found in milk, meat and food products and said to be major cause of food poisoning and many other illness. This study concluded that *E. coli* O157:H7 is prevalent in poultry meat in our country as a great hazard to public health. PCR is sensitive, specific and efficient way for confirmation of *E. coli*. as compared to bacteriological and biochemical methods.

Recommendations

This microbiological study will help other researchers to address meat borne diseases/illnesses, which are considered as major threat to public health especially in developing countries like Pakistan. Due to the public health significance of *E. coli O157:H7*, necessary hygienic measures are suggested to reduce or prevent the chance of infections from poultry meat. Furthermore, awareness campaigns concerning importance of hygiene measures should be encouraged to reduce or prevent the chance of infection and to aware the public from food borne health risks. Public health authorities should come forward for the continuous monitoring and strict implementation of public health policies by World Health Organization(W.H.O) at national level.

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