Avian Pathogenic Escherichia coli (APEC) in Palestine:

Characterization of Virulence Factors and Antibiotic Resistance Profile

Mohammad Qabajah[1] and Yaqoub Ashhab[1]

[1]Biotechnology Research Center, Palestine Polytechnic University
Hebron, Palestine
Mohammad_qabaja@ppu.edu; yashhab@ppu.edu

ABSTRACT

Escherichia coli bacterium is common to many environments and there are over 150 different strains. Avian pathogenic *E. coli* (APEC) strains cause diseases in birds at various ages. The introduction of such strains to chicken respiratory tract causes invasive infections, collectively known as colibacillosis. It can cause extensive mortality in poultry flocks leading to great economic losses. Recent reports showed that the APEC pathogenicity is associated with certain virulence genes (*papC*, *astA*, *vat*, and *irp2*) are located within the bacterial genome and/or their ColV plasmids (*tsh*, *iucD*, *iss*, and *cvi*). Identification and characterization of these genes are essential to implementing efficient disease control and prevention systems. The aim of this study is to identify the virulence associated genes and the antibiotic resistance profiles of APEC strains in Palestine.

Internal organ samples from 83 infected flocks were collected and tested for presence of the mentioned virulence genes using an adapted and improved multiplex PCR protocol. The resistance of the isolated strains to 10 commonly used antibiotics in Palestine was analyzed using the disc diffusion method. The multiplex PCR of the tested samples revealed a high prevalence of the following genes: *iss* and *cvi* 100%, *astA* 98.48% and *iucD* 78.79%. The genes *vat* and *papC* have a prevalence of 34.85% and 31.81%, respectively. To a lesser extent *irp2* 19.70% and *tsh* 10.61% were identified. The study of antibiotic susceptibility profiles showed high resistance levels against Tetracycline 100%, Ampicillin 83.33%, Amoxicillin 83.33%, Kanamycin 80.3%, Ciprofloxacin 72.72% and Neomycin 69.70%, while the lowest resistance levels were against Nitrofurantoin 18.18% and Cephalexin 12.12%.

The improved multiplex PCR has proven to be a useful and rapid assay to identify virulence factor profiles of APEC. In Palestine, the indiscriminate use of antibiotics should be avoided. It may increase the risk of development of drug-resistant *E. coli* strains that constitute a human risk due to zoonose potential reservoir of Extended-spectrum b-lactamases resistance genes. Therefore, programs are recommended to increase farmer's awareness about the devastating effects of antibiotic misuse. In addition, the authorities must take a responsible role through imposition a set of regulations to ensure safe poultry products

Keywords; APEC, multiplex PCR, antibiotics, virulence genes.

INTRODUCTION

Escherichia coli belong to the Enterobacteriaceae family; these bacteria include a broad variety of different types including pathogenic and nonpathogenic strains. *E. coli* usually colonizes the gastrointestinal tract of most warm-blooded animals as a part of the normal intestinal flora[1-2]. Some strains can be highly pathogenic and may cause serious problems especially in immuno-compromised hosts causing intestinal and extraintestinal infections[3]. In humans, *E. coli* is responsible for three types of infections: urinary tract infections, neonatal meningitis, and intestinal diseases [4].

Avian Pathogenic *E. coli*

Pathogenic *E. coli* strains are also related to extraintestinal infections for other animals [5]. Among birds, it was proved that the pathogenic strains of *E. coli* cause respiratory diseases [6-8]. Ten to fifteen percent of the intestinal coliforms in chickens have a potential to be pathogenic [9]. Avian pathogenic *E. coli* (APEC) strains are mainly associated extraintestinal diseases [1, 7] and responsible for great losses in the avian industry [6, 8, 10]. Colibacillosis is the most important disease caused by APEC strains and is characterized by multi-extraintestinal disorders [9, 11], birds from 2 to 12 weeks of age are more susceptible to disease and mortality rates may reach as high as 20% in birds within the 4 to 9 weeks age range [1].

Identification Methods of Avian Pathogenic *E. coli*

APEC identification has passed through a number of developments, beginning with conventional methods of identification based on chemical characterization and the nature of selective media. A more specific identification approach is the serotyping analysis that aims at classifying pathogenic strains based on their surface antigens. The DNA based identification methods are considered as extremely sensitive approaches that aim at the identification of *E. coli* virulence genes.

Recent researches mentioned that *E. coli* serotyping is not efficient enough for APEC classification [6, 11-12] especially as some of the available commercial kits even they are very expensive, do not include all O antisera which lose the ability to classify all the O serotypes. In some cases, O serotyping was not able to classify around 50% of total APEC strains [11, 13]. Thus, serotyping identification will not be able to give us clear information about the pathogenic strains of *E. coli* and it is not able to classify all of these strains.

The limited knowledge about the molecular epidemiology as well as about virulence-associated genes of avian pathogenic *E. coli* (APEC) hinders the application of essential and efficient epidemiological control measures for the prevention of colibacillosis. However, several studies have identified genes encoding virulence factors of APEC. The according genes encode adhesion-related factors such as P-fimbriae (*papC*) and

a temperature-sensitive hemagglutinin (*tsh*); iron-acquisition systems (*fyuA/irp2*) and aerobactin (*iutA/iucD*); a protein for increased serum survival (*iss*); a colicin V plasmid (*cva/cvi*); an enteroaggregative heat-stable toxin (*astA*); as well as a vacuolating autotransporter toxin (*vat*).

APEC and food safety

Due to the low cost of production as well as the relatively cheap prices, poultry meat is very important in the consumer market including the Palestinian one. However, epidemiological reports showed that the presence of pathogenic and spoilage microorganisms in poultry meat and its by-products remains a significant concern. *E. coli* has been consistently associated with food-borne illnesses in most countries of the world [14].

Recent studies have suggested that some APEC strains are considered as potential zoonotic agents [15-17]. Earlier works had shown APEC strains to be easily transmitted to humans [18-19]. Indeed, studies have shown that some APEC strains could belong to the same clones as human ExPEC strains [20-21]. Recently, it has been reported that very closely related clones of serotype O18:K1:H7 could be recovered from extra-intestinal infections in humans and chickens and that isolates from both species were virulent for chicks [22]. PCR-based phylotyping and multi-locus sequence typing have revealed a link between APEC and human ExPEC [16, 23], further suggesting the potential food-borne source of human ExPEC. Consistent with these observations, whole genome sequence analysis has revealed a high degree of similarity between APEC and ExPEC, with only 4.5% of the APEC O1:K1:H7 genome not found in three ExPEC genomes [23].

In Palestine, colibacillosis is a common disease responsible for hundreds of thousands shekels losses for Palestinian economic sector every year. At farmer level, there is unregulated and uncontrolled use of antibiotics to control this disease irrespective of any negative consequences that may result of antibiotic misuse.

Extended-spectrum ß-lactamases

Extended-spectrum ß-lactamases (ESBLs) are a group of enzymes can hydrolyze a variety of ß-lactams including cephalosporins and penicillins. ESBL-producing bacteria become resistant to wide range of such ß-lactams. Recently, ESBL-producing organisms are common among hospitals as healthcare associated pathogens [24]. *E. coli* is the most recognized in such cases. ESBL-producing *E. coli* has a potential to become a major public health problem in the near future.

ESBLs in *E. coli*

TEM- and SHV-derived ESBLs were the commonly observed ESBL types in *E. coli* [25]. On the other hand, a newer group of ESBLs called CTX-M showed the ability to hydrolyze the cefotaxime over ceftazidime in *E. coli*. Currently, five groups of CTX-M-type ESBLs have been identified: CTX-M-1, -2, -8, -9 and -25 groups [26].

MATERIALS AND METHODS

Samples

Samples from 83 broiler farms suspected to have *E. coli* infection were collected between Feb-Jun 2009 through the Central Veterinary Laboratory - Ramallah - Palestine. The farms were distributed over 56 locations from 11 cities in West-Bank Palestine. The samples were internal organs that are usually obtained in the routine postmortem examination of birds suspected to have died from colibacillosis infection.

APEC reference strain

The international APEC reference genomic DNA of the strain O78χ7122 was provided by Dr. Francis Dziva from the Institute of Animal Health – Compton – UK. This strain contains the virulence genes: *astA*, *iss*, *papC*, *iucD*, *tsh*, and cvaC. Therefore, it was used as positive controls to establish the multiplex PCR protocol.

Culture and biochemical characterization

E. coli Broth with MUG (OXOID) was used to improve *E. coli* detection. Internal origins of suspected liver were inoculated in *E. coli* Broth with MUG (EC-MUG broth) at 44.5ºC for 22 hours to 26 hours as an *E. coli* specific pre-enrichment step. In the presence of 4-methylumbelliferyl- β-D-glucuronide (MUG), *E. coli* produces the enzyme glucuronidase that hydrolyzes MUG to yield a fluorogenic product which is detectable under long-wave (366 nm) UV light.

Isolates were then cultured into 7% sheep blood agar, MacConkey agar and eosin -methylene blue agar (OXOID). The identification of *E. coli* was based on the results of diagnostic tests, which includes gram stain, colony characterization, gas production and ability to be enriched in the EC-MUG Broth. All confirmed strains were kept at -80 ºC in EC-MUG medium containing 15% glycerol. Further molecular identification was performed by using specific designed *E. coli* primers for beta-D-glucuronidase gene (uidA) uidA-F: CTGAACTGGCAGACTATCCC and uidA-R: CAGCACATCAAAGAGATCGC.

Antimicrobial sensitivity

A sensitivity test for ten antimicrobial agents was carried out on the isolated strains by the standard disk procedure [27] on Muller-Hinton agar. Ampicillin, Tetracycline, Amoxicillin, Neomycin, Gentamycin, Nitrofurantoin, Ciprofloxacin, Kanamycin, Chloramphenicol and Cephalexin standard paper disks were laid on the medium. The plates were incubated for 24h at 37ºC and inhibition zones were measured. In addition, the distribution of the resistant isolates for the ten antibiotics was tested to study if there was any relationship between the resistances of these isolates and the geographical location were taken from.

Multiplex PCR analyses

A multiplex PCR protocol was adapted and improved to detect the presence of the following virulence genes: *papC*, *astA*, *vat*, *irp2*, *tsh*, *iucD*, *iss*, and *cvi*. The gene specific primers (Table 1) were designed using PerlPrimer v1.1.19 which is open-source primer design software (perlprimer.sourceforge.net). Each primer was tested for the following criteria: internal stability, melting temperatures, cross dimerization with the other primers and non-specific binding either to *E. coli* or to the host genome.

Bacterial colonies from overnight MacConkey agar at 37ºC were picked using a sterile pipette tip and aseptically suspended in 100 ul of sterile distilled water in an eppendorf tube. The suspension was boiled for 15 min. After centrifugation for 5 min at 13000 r.p.m, 2ul of the supernatant were taken as template DNA and added to the PCR reaction mixture (50ul) containing 0.5ul of each primer (10 pmol concentration), 4ul of the four deoxynucleoside triphosphates (10mM solution), 5ul of 10X PCR buffer, 8 ul of 20 mM Magnesium Chloride, and 5 units of Taq-Polymerase. The samples were subjected to 30 cycles of amplification. The cycling conditions were as following: step1, 5 min at 94ºC; step2, 1 min at 94ºC; step3, 45 sec at 55ºC; step4, 2 min at 72ºC (step2–step4, repeated 30 times); step5, 10 min at 72ºC.

The amplification products were analyzed by electrophoresis on 2.0 % agarose gel, in 1x TBE buffer for 90 min at 90V. The amplicons were stained with ethidium bromide, and photographed under illumination (UV box from UVP, United States).

The geographical distribution of the eight genes has been tested also to detect if there is any possible links with the presence of such genes in specific geographic locations.

Table 1: Gene specific PCR primers used in this study

Gene	Primer	Primer sequence (5'- 3')	Localization within gene	$T_{melting}$ (Cº)	Amplicon size (bp)
cvi	*cvi(F)115*	CCATGCATACATTTTGCTTCTCTG	115 – 138	63	114
	cvi(R)229	AGTCAGAGTTCTCATATGATCTCC	229 – 206	61	
iss	*iss*(F)102	GCAGTAACACCAAAGGAAACC	102 – 122	62	184
	iss(R)286	CTTCCAGCGGAGTATAGATGC	286 – 266	63	
astA	*astA*(F)249	GATCCCTGGTACAACTATCGC	249 – 269	62	266
	astA(R)515	TAGCCGTGTTCGTCAATCAC	515 – 496	63	
iucD	*iucD*(F)315	GCTGCTGAAGATATGAATAACC	315 – 336	60	431
	iucD(R)746	CGAATATCTTCCTCCAGTCC	746 – 727	60	
papC	*papC(F)857*	CTATGCACCGCAGATTACC	857 – 875	61	538
	papC(R)1395	GAACGTAATGTCGGCATCC	1395 – 1377	60	
vat	*vat*(F)824	ACTGGTCGGTGTTTACTCG	824 – 842	62	682
	vat(R)1506	GTCATTCCCGTTAACATCCAG	1506 – 1486	61	
tsh	*tsh*(F)1341	GTTGTACTGAACCAGCAGG	1341 – 1359	61	786
	tsh(R)2127	GTTCTTCAGTGACAGCCTG	2127 – 2109	61	
irp2	*irp2*(F)2048	GTCAGACGATATTCCCGTCC	2048 – 2067	62	886
	irp2(R)2934	CAGCTCGATGCGATATCCTC	2934 – 2815	63	

ESBL Detection

0.5 McFarland standard bacterial suspension was inoculated on Mueller-Hinton agar for disc diffusion testing. amoxicillin-clavulanic acid disc was placed towards plate center. ceftazidime (CAZ) disk was placed so that its inner edge is 15 mm out from the edge of amoxicillin-clavulanic disc at 90o angles. The same with cefotaxime (CTX), ceftriaxone (CRO) and cefpodoxime (CPD) discs so that they were spaced 90o apart and 15mm from the center disc. cefoxitin disc was placed in any available space remaining on the plate. Disc was incubated for 18-24 hours at 35oC in O2 presence; the zone diameters for the all cephalosporins were measured. This will enable the phenotypic screening and group classification of ESBLs producer strains. For molecular confirmation, universal specific primers were used for blaCTX-M genes detection in *E. coli*, CTX-M-UF forward primer: ATGTGCAGYAGGACTAA and CTX-M-UR reverse primer: CCGCTGCCGGTYTTATC.

RESULTS

Identification of *E. coli* isolates

Out of the eighty three field samples, sixty six were positive for *E. coli* based on morphological and biochemical characteristics. Using gram stain method, the sixty six positive isolates showed short-rod gram negative bacteria. The sixty six isolates showed pink colonies on MacConky agar and green metallic sheen colonies on EMB agar, which are typical for *E. coli*. In addition, all of the isolates were able to be enriched in EC Broth-MUG and this is represented by their capability to produce fluorogenic product that is characteristics to E. coil.

Antibiotic susceptibility testing

All of the *E. coli* isolates showed resistance to at least two or more antibiotics. The susceptibility pattern of the antimicrobial agent tested showed different levels of resistance. The first group includes the antibiotics to which there were high levels of resistance (69% to 100%); these are Tetracycline (TE): (100%), Ampicillin (AMP): 83.33%, Amoxicillin (AML): 83.33%, Kanamycin (K): 80.3%, Ciprofloxacin (CIP): 72.72% and Neomycin (N): 69.70%). The second group includes the antibiotics to which there were moderate levels of resistance (30% to 69%); these are Gentamycin (CN): 50% and Chloramphenicol (C): 39.39%. The third group includes the antibiotics to which there were low levels of resistance (0% to

30%); these are Nitrofurantoin (F): 18.18% and Cephalexin (CL): 12.12% (Table2).

No relationship between the prevalence of resistant isolates to antibiotics with the geographic locations where the isolates from, as the prevalence distribution of these resistant isolates were consistent in most geographical locations

Further exploration the association between resistance to the used antibiotics to reveal possible resistance patterns. Table2 shows nine different patterns of the sixty six *E. coli* isolates. Pattern number 1 (TE/AMP/AML/N/CIP/K) was the most common pattern (43.94%) and pattern number 8 and 9 (TE/AML/N/CIP/K) and (TE/AMP) showed the least common patterns (3.03%).

Table 2: Antimicrobial sensitivity of *E. coli* isolates

Resistant level	Antibiotics	Number of resistant isolates (%)
High level	Tetracycline (TE)	66 (100)
	Ampicillin (AMP)	55 (83.33)
	Amoxicillin (AML)	55 (83.33)
	Kanamycin (K)	53 (80.30)
	Ciprofloxacin (CIP)	48 (72.72)
	Neomycin (N)	46 (69.70)
Moderate level	Gentamycin (CN)	33 (50.00)
	Chloramphenicol (C)	26 (39.39)
Low level	Nitrofurantoin (F)	12 (18.18)
	Cephalexin (CL)	8 (12.12)

Virulence genes

The efficiency of the multiplex PCR versus the gene-specific PCR reactions was further assessed using 12 isolates selected randomly from the 66 positive samples. The results of this comparison were confirmatory and they showed almost identical pattern of virulence genes in both PCR protocols.

In order to rule out the mixed infection by more than *E. coli* strain, a pilot experiment was performed for 15 isolates. For each isolate, six colonies were picked from the enriched culture and were tested individually by the multiplex PCR. The results showed that the 6 colonies of each isolate have the same genetic profile for the 8 virulence factors.

The optimized multiplex PCR reaction was used to screen the whole panel of the 66 isolates. (Figure 1) represents typical gel electrophoresis results of the multiplex PCR for a group of isolates used in this study.

Two virulence genes; *iss* and *cvi* showed the highest prevalence (100%), while *astA* (98.48%) and *iucD* (78.79%) were less prevalent. The genes *vat* (34.85%) and *papC* (31.81%) were present in about one third of the tested strains. To a lesser extent multiplex PCR identified *irp2* (19.70%) and *tsh* (10.61%) (Table3). Despite the differences in the geographical source of these isolates,

The distribution of the pathogenic genes that have been tested was consistent among included isolates.

More than 91% of the isolates showed four virulence genes at least, four virulence associated genes are considered as minimal cut-off number of genes for avian *E. coli* to be considered pathogenic strain [28]; all strains possessed two virulence genes at least.

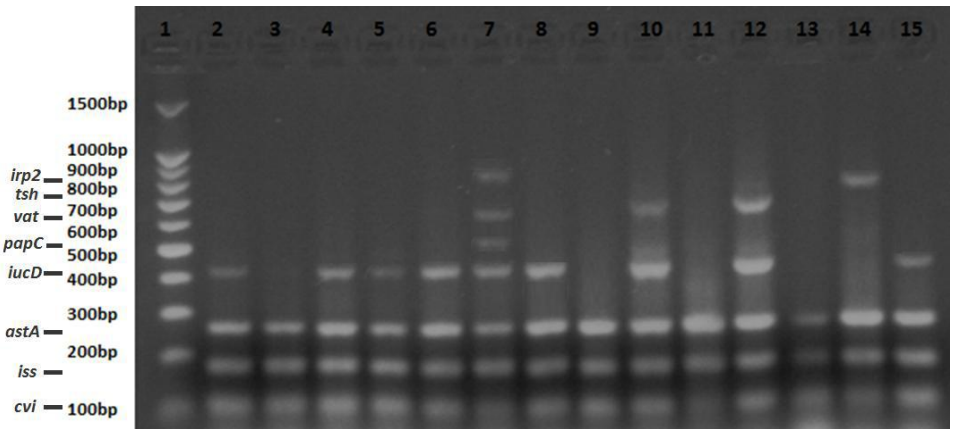

Fig. 1: Agarose gel electrophoresis of the multiplex PCR products with representative APEC isolates carrying various combinations of virulence determinants. Lane 1: 100bp DNA ladder; lane 2: Isolate 022QH; lane 3: Isolate 021HN; lane 4: Isolate 024JN; lane 5: Isolate 027BM; lane 6: Isolate 028RH; lane 7: Isolate 014RH; lane 8: Isolate 030RH; lane 9: Isolate 023RH; lane 10: Isolate 016HN; lane 11: Isolate 029HN; lane 12: Isolate 020ST; lane 13: Isolate 054NS; lane 14: Isolate 052HN; lane 15: Isolate 033BM.

Table 3: Prevalence of virulence-associated genes in APEC isolates included in this study as detected by multiplex PCR

Tested strains	Immunity		Adhesion	Toxin		Serum survival	Iron uptake	
	cvi	***papC***	***tsh***	***astA***	***vat***	***iss***	***iucD***	***irp2***
n = 66	66	21	7	65	23	66	52	13
%	100	31.81	10.61	98.48	34.85	100	78.79	19.70

EBSL

Out of 66 isolates, 7 isolates (10.61%) were ESBL positive depending on double disc diffusion method, the 7 isolates showed a potentiation of the inhibition zone for at least one of 3rd generation cephalosporin used when combined with clavulanic acid, and all these isolates were susceptible to cefoxitin. To be classified as class A ESBL. At the molecular level of blaCTX-M gene identification, 8 isolates (12.12%) were positive for CTX-M gene.

DISCUSSION

Samples Identification

In the present study 83 samples of colibacillosis suspected broiler chicken were used to study and characterize avian pathogenic *E. coli*. 66 samples showed positive for *E. coli*, while the other 17 samples were negative. The lack of *E. coli* in these samples can be due to different reasons. After a recheck of the farms' history, it was noted that many of these samples were obtained from farms that had been subjected to antibiotic treatment directly after the onset of the disease, it is known that antibiotic treatment prevents the isolation of pathogens in most cases. On the other hand, it is known that the pathogenicity of *E. coli* passes through several stages and the last stage is the colonization of the internal organs, since internal organs were the source of isolates, it is possible that the disease itself has not reached the advanced stages, this means that the bacteria did not reach the colonization level at the internal organs. This may have contributed to inability to obtain the bacteria due to its absence from the sample tissue. Also, other pathogens rather than APEC may have caused similar diseases which explain the absence of APEC.

Enrichment step

In this study, the specimens that were initially obtained from internal organs were inoculated in a special EC Broth medium that contains 4-methylumbelliferyl- β-D-glucuronide (MUG). The EC-MUG medium was used for its ability to selectively enrich *E. coli* bacteria due to the special additives that it contains. This medium consists of lactose as carbon source, 0.15% bile salts as a selective agent against gram positive bacteria, and 4-methylumbelliferyl- β-D-glucuronide (MUG) compound which can be hydrolyzed by glucuronidase enzyme produced by *E. coli* to yield a fluorogenic product which is detectable under long-wave (366 nm) UV light. While *E. coli* bacterium is a gas producer bacteria and has the ability to grow at 44.5ºC, the enrichment of *E. coli* in EC-MUG at 44.5ºC for 22 to 26 hours with fluorescence and gas production is considered as specific indicator for *E. coli* enrichment Selection may have affected the results by eliminating other similar bacteria such as shigella which may share genetic markers with APEC. The specific enrichment and selection of *E. coli* in our study was necessary to reduce the false positive results that may appear from other gram negative bacterial species such as Salmonella Typhimurium, and Shigella flexneri which may harbor the same ColV plasmid.

Multiplex protocol

In order to ensure good performance of multiplex PCR, PerlPrimer v1.1.19 software was used to examine essential primer criteria. All primers were analyzed carefully to optimize the melting temperatures, secondary structures, self and cross dimerization, and homology to non-specific genomic template either from *E. coli* itself or from host genome. Using primers that were used in other similar studies was deliberately avoided unless they were carefully analyzed for the mentioned criteria. Some primer pairs that were used in other studies have dissimilar annealing temperature reaching 13ºC and some of these primers suffer from a strong cross dimerization which might affect amplification efficiency[28].

Molecular identification

This study targeted 8 virulence genes that are typically associated with APEC and they are grouped into five functional classes; genes essential for adhesion (*papC* and *tsh*), toxin genes (*astA* and *vat*), serum resistance (*iss*), iron uptake (*irp2* and *iucD*), and *cvi* gene which protect *E. coli* against bacteriocin [28].

The eight virulent genes were present in different combinations ranging from two genes in some isolates to seven genes in other isolates. It is interesting to notice that the pattern of virulent genes in each isolate tends to represent the different functional classes. This might indicate that these genes work in integrated manner to ensure the ability of bacteria to survive and to move from one stage to the next until a full bloom colibacillosis.

The *cvi* gene was detected samples that were *E. coli* positive. The importance of this gene is that it confers the immunity to bacteria against a specific bacteriocin as it encodes the colicin V immunity protein (Fath et al., 1991). In ColV plasmid containing bacteria, colicin V immunity protein can protect the cell against colicin V encoded and secreted by a dedicated export system[29].

Adhesion to the lung cells of the bird is essential to APEC especially during the early stages of infection. Pilus is the main virulence factor involved in adhesion of pathogenic *E. coli* to the host cells including: type 1, P, and curli pilus [30]. The subunit C of type 1 pilus is encoded by fimC gene, and *papC* gene. *papC* is the main functional gene of P pilus. This gene was identified in 31.81% of the sixty six *E. coli* isolates. This frequency is in agreement with previous work which showed (30.0%) [31]. Compatible to a previous work by Ngeleka et al which showed (15.4%) [32]; only 10.61% of isolates in this study were positive for *tsh* gene, which is another adhesion-related factor[33]. This might indicate that the presence of *tsh* gene is not necessary to increase the level of pathogenicity of APEC. It is interesting to notice that the 62% of the examined isolates neither have *papC* nor *tsh* adhesion genes. This does not mean that these isolates do not contain any gene coding for adhesion factor. In fact there are several adhesion associated genes that were not examined in this study. Therefore, it is, possible that such isolates contain one or more of the unexamined adhesion factors. This opens a new avenue for

investigating the adhesion factors and tissue tropism association.

Iron acquisition systems have been recognized to be associated with bacterial virulence especially in bacteria causing septicemia[34-35]. 80.30% of isolates have at least one of the two examined iron acquisition encoding genes *iucD* and *irp2*.This result demonstrates, indeed, the importance of iron acquisition systems during pathogenesis. In fact, bacteria depend on these systems to get the heme molecule to survive in their host and in aquatic habitats. Remarkably, *iucD* gene that belongs to the aerobactin iron acquisition system stands as the major factor for regulating iron uptake in *E. coli*. It was also described as part of the PAI Shigella island-2 [36]. It was found that 79% of the examined APEC isolates were positive for *iucD* gene. This result is in agreement with several reports which demonstrated that most APEC strains (63% – 98%) express the *iucD* aerobactin iron acquisition system[6, 37].

In this study, all isolates were positive for increased serum survival gene (*iss*). Johnson et al demonstrate that *iss* is significantly associated ($p < 0.0001$) with APEC strains than nonpathogenic strains and may be an indicative of its ability to cause disease[17]. Three alleles of *iss* gene have been identified; one of them is harbored in the ColV virulence plasmids and the others two are chromosomal [17]. Thus, *iss* has a vital role in *E. coli* pathogenicity and could be a potential target for developing novel therapeutics and prevention strategies.

Secretory toxins play key role in enabling pathogenic *E. coli* to influence the biological processes of the host [38]. In *E. coli* samples, 98.48% were positive for the *astA* toxin gene. This gene encodes for a peptide enterotoxin-1sequence that is heat-stable, which is a member of heat-stable secretory enterotoxins (STs). STs remain active at temperatures as high as 100oC while they can keep their 3D structure in such high temperatures [39-40]. STs can recognize different receptors on the surface of host cells and affect different intracellular signaling pathways.

About 35% of the isolates were positive for *vat* gene. Vacuolating autotransporter toxin (Vat), the product of *vat* gene, which plays a major role in protein hydrolysis as a result of serine-type endopeptidase activity [41]. Vat has been shown as a factor involved in the pathogenicity of APEC strains [6, 28, 41]. In addition to its adhesion related function, Tsh protein which is the product of *tsh* gene, shows a serine-type endopeptidase function similar to Vat [41].

Antibiotic susceptibility

Comparing data with studies performed in Europe, United States and Japan [42-44] Palestinian isolates were relatively more resistant for antibiotics than European, United States, and Japan isolates.

In Palestine, the Central Veterinary Laboratory confirmed that there was an exaggerate misuse of antibiotics in the poultry sector, which may have enhanced antibiotic selection of resistant strains in various zoonotic pathogens. Unfortunately, there were no studies about the actual consumption rates of antibiotics in the Palestinian poultry sector. However, comparing obtained results of antibiotic resistance with the information available at the Central Veterinary Laboratory, showed a positive correlation between the consumption and resistance frequency. For example, farmers rarely use Nitrofurantoin and Cephalexin which show a good antibacterial activity, whereas the majority of isolates were fairly resistant to Tetracycline, Ampicillin, Amoxicillin, Kanamycin, and Ciprofloxacin, which are themselves or their closely related antibiotics are commonly used (CVLs).

The indiscriminate use of antibiotics in Palestine may have a major role in increasing resistance in *E. coli*, which has a distinctive ability to adapt with its surrounding environment. Through field trips to more than 15 poultry farms it was observed that all visited farms were routinely subjected to various types of antibiotics as “a preventive prophylactic measure” as they said.

In addition to the misuse of antibiotics, the lack of biosafety standards during rearing and transporting birds can play a major role in accelerating the development of resistance in *E. coli* as well as many other pathogenic organisms. For example, the lack of proper sterilization for the farm between the consecutive breeding cycles, the poor hygienic conditions, and malpractices during preslaughter handling and transportation can create an ideal environment for the genetic transfer of antibiotic resistant genes within and across species [45].

E. coli is one the main reservoirs of moveable elements of antibiotic resistance. The high capacity of these bacteria for horizontal gene transfer poses a clear danger for the antibiotics future [46]. The ability of these bacteria is not limited to transfer of the genetic contents into other *E. coli* strains; in fact, *E. coli* is a common member of biofilms where many species of bacteria exist in close proximity to each other allowing genetic exchange of antibiotic resistance including multidrug resistant plasmids to other bacteria. Subsequently recipient bacteria gain the ability to resist many kinds of antibiotics[47-49].

Recently, many of extended-spectrum beta-lactamases (ESBL) producing *E. coli* have become a world-wide problem. The ESBL-positive *E. coli* strains are highly resistant to a broad range of antibiotics. Controlling such strains with commonly used antibiotics is ineffective; currently there are very few antibacterial alternatives that remain effective against these multi-resistant pathogens[50-51].

It is very important to control APEC because it represents a grave danger to domestic animals and is a potential source of transferring multi-drug resistance genes to human specific *E. coli* or other bacteria such as Staphylococcus aureus and shigella strains [28, 47, 52]. The fact that this pathogen is naturally present in daily consumed food should be considered as a serious public health and food biosafety issue.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The improved multiplex protocol is able to detect eight of APEC pathogenicity related genes to give a picture regarding the virulence factors profile

The minimal cutoff number of virulence factors is four factors; 91% of the identified isolates had 4 or more virulence factors.

Most of the identified isolates are multidrug resistance to different types of antibiotics screened in the study

Recommendations

Because the poultry sector in Palestine is a very important sector on the health and economic levels, there is a vital need to monitor and develop this sector through concerted efforts,

especially between the involved responsible authorities, farmers, and consumers.

Authorities can enact different roles to support such areas economically and to meet the farmers' needs. Authorities can plan research studies on the national level, which puts Palestine on the map and determine the level of agriculture sector in Palestine relative to international standards.

Farmers have to follow rules based upon scientific consultation in poultry farming and antibiotic use.

Consumers have the right to obtain healthy food sources that are certified by the Ministry of Health.

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