CRIPSR-Cas9 mediated *waaL* gene excision of the Avian Pathogenic *E. coli* (APEC) for its pathogenicity evaluation

By

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(NUST 2017ASAB)

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Islamabad, Pakistan

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A thesis submitted as final year project as a requirement of MS

In

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DECLARATION

I certify that this research work titled **"CRIPSR-Cas9 mediated *waaL* gene excision of the Avian Pathogenic *E. coli* (APEC) for its pathogenicity evaluation"** is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

Quratul Ain

Master of Science in Industrial Biotechnology

Registration No. 00000203969

Dedication

I can name many turning points in my life that I believe changed me, but when I sit back and think about it there is one particular time in my life that changed me for good. It was the time when I started working with molecules, microbes and intrication between them I got time to think about life and its underlying behaviors itself. I learnt how molecules interact together by making new bonds for the good and they break apart in order to get rid of the harmless ones, how they transport and make stronger each other when and where needed the most and take them away from where they are least required, how do they co-operate to bring a change by presenting an example of exception and unification. This made me realize that what are we? Nothing but a collection of cells, neurons, nerve endings, synapses where in the single and double helix dwells. We all are evolved to self-replicate goodness and positive vibes if and where needed, detaching ourselves from negativity and toxicity instead of indulging it within our self thus we all are a biological machine, the unique self , the work of art do not just teach us biology, they teach us to being a human.

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Summing up my journey of research at ASAB I would like to quote the words of Sir Winston Churchill: “Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.”

Quratul Ain

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List of Abbreviations

APEC	Avian Pathogenic *Escherichia coli*
bp	base pair
Cas9	CRISPR associated protein 9
CHL	Chloramphenicol
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
DSB	Double Stranded Break
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
ExPEC	Extra-intestinal Pathogenic *Escherichia coli*
GIT	Gastrointestinal tract
gRNA	Guide RNA
HDR	Homology directed repair
KAN	Kanamycin
kb	kilobase
LB	Luria Broth
LPS	Lipopolysaccharide Synthes
MDR	Multi-Drug Resistant
N-Agar	Nutrient Agar
N-Broth	Nutrient Broth
NCBI	National Center for Biotechnology Information
NHEJ	Non-homologous end joining
NMEC	Neonatal Meningitis *Escherichia coli*
O.D	Optical Density
P. aeruginosa	*Pseudomonas aeruginosa*

PAM	Protospacer Adjacent Motif
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
Spp.	*Specie*
TALEN	Transcription activator-like effector nuclease
tracrRNA	Trans-activating crRNA
Tris HCL	Trisaminomethane Hydrochloride
TSB	Trypton Soy Broth
UPEC	Urinary Pathogenic *Escherichia coli*
UV	Ultra-Violet
ZFP	Zinc finger protein

Abstract

Avian Pathogenic *Escherichia coli* (APEC) is considered amongst the leading causes of colibacillosis in poultry flocks and it is responsible for nearly 18% mortality in poultry which leads to significant economic loss to the poultry industry every year. The study focuses on employment of a new antibacterial strategy, in order to combat colibacillosis caused by multi drug resistant APEC strain. This study aims on the effective gene knockout of the lipopolysaccharide (LPS) synthesis gene "*waaL*" via CRISPR/CAS9 system, in order to assess its effect on pathogenicity of APEC. Sequence of suitable gRNA of 24bps was designed in the functional part of the gene based on GC content, minimal off-target sites and PAM sequence. The gRNA was ligated in the pCRISPR and along with pCas9 both were successfully transformed in the APEC wild type strain. The mutant strains M1-O1 and M12 -O1 along with WT-O1 were sent for sequencing for confirmation of gene knockout followed by several *in vitro* pathogenicity evaluation tests. Under different physiological stress conditions, mutants M1-O1 and M12-O1 were found to be more sensitive to heat shock, alkali, acid and osmolarity shock as compared to WT-O1. Furthermore, WT-O1 have the potential to survive and grow in urinary bladder and might have zoonotic potential as well whereas on the other hand M1-O1 and M12-O1 showed decreased growth in human urine as compared to WT-O1 thus indicating *waaL* gene might be involved in pathogenicity. Antibiotic susceptibility testing (AST) was performed, where strains were evaluated against 22 most commonly used antibiotics and the results showed that M1-O1 and M12-O1 showed resistance against more antibiotics when compared with WT-O1. Biofilm quantification and motility assays were performed which showed reduction in biofilm forming ability of M1-O1 and M12-O1 and reduction in swimming and swarming motility of M1-O1 and M12-O1 in comparison with WT-O1 thus proposing the role of *waaL* gene in bacterial virulence and pathogenicity of APEC.

1. Introduction

E. coli belongs to Enterobacteriaceae family having rod like shape and a gram-negative bacterium. The bacteria inhabit in the normal intestinal microflora as commensals and keeps the gut healthy. However, by acquiring virulence factors through plasmids, transposons, pathogenicity islands and bacteriophages *E. coli* become pathogenic and can cause serious illness (Tenaillon et al., 2010).

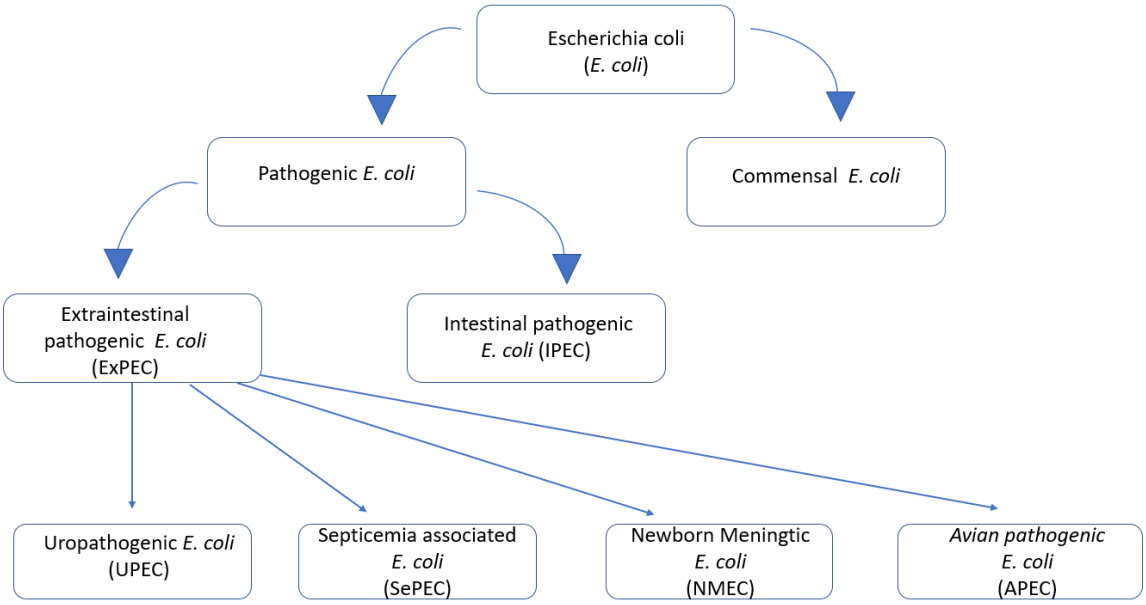

Figure 1.1 Schematic representation of *E. coli* groups and its pathotypes

E. coli that are pathogenic have two classes first one is (IPEC) (Intestinal pathogenic *E. coli*) or diarrheagenic *E. coli* and ExPEC (Extraintestinal pathogenic *E. coli*) (Croxen & Finlay, 2010). Unlike diarrheagenic *E. coli*, ExPEC are unable to cause diseases in the GI. However, ExPEC is the causative agent of various infections at various anatomical sites outside the gastrointestinal tract (Kucheria et al., 2005). By passing the intestinal epithelium, it can colonize other organs such as Urinary tract, blood and brain where it causes diseases like UTI, septicaemia and neo natal meningitis respectively in humans while in birds it causes colibacillosis (Clermont et al., 2013).

APEC is an etiologic agent of extra intestinal infections in chicken collectively known as colibacillosis. This disease serves as one of the main reasons of significant mortality and mobility followed by economic loss in poultry sector. The risk factors associated with colibacillosis include cellulitis, coli granuloma, omphalitis, swollen head syndrome, enteritis, peritonitis, pericarditis, airsacculitis, septicaemia leading towards the death of chicken. In Pakistan, especially in Punjab which has majority of country's poultry farms, 10.1% prevalence of colibacillosis was reported (Rehman et al., 2013). Whereas a study conducted earlier in 2012 stated colibacillosis as the second most prevalent poultry disease having a prevalence rate of 18.61% (Ahmad et al., 2012).

It is noteworthy to mention that genome of *E. coli* is very diverse as it is comprised of housekeeping genes that play vital role in the survival of bacteria, resistome conferring resistance to antibiotics, pathogenicity island (Raimondi et al., 2019) and there are number of genes whose pathogenicity still needs to be confirmed. Lipopolysaccharide (LPS) forms the outside membrane of bacteria that are gram-negative, which acts as an endotoxin and LPS plays a vital part in the structural integrity and providing protection to bacterial cell (Alexander & Rietschel, 2001).

There are number of genes that are linked with bacterial metabolism, adhesion, invasion, regulation and LPS biosynthesis that have been reported to play significant function in pathogenicity of APEC (Janßen et al., 2001). There are 3 parts that mainly compose: lipid A, core oligosaccharide and O-antigen. The studies reveal that there are different genes that synthesize the O-antigen (Wang & Quinn, 2010). The colonization of bacteria and killing via complement-mediation are distinctively done with O-antigen. The O-antigen is also transported through the lipid-A core ligase (*waaL*), O-antigen flippase (wzy), O-antigen regulator of chain length (wzz) as well as transporter of O-antigen (wzx). Other than transportation they also help in maintaining LPS O-antigen's structure (Wang & Quinn, 2010).

Earlier studies revealed that the O-antigen's process of ligation towards the surface of LPS is one of the crucial steps. The O-antigen which is preassembled is ligated to core of lipid A, via the involvement of product produced by *waaL* gene. It is also very vital for synthesizing (Heinrichs et al., 1998). *waaL* gene has been reported to be encoding O-antigen ligase. The oligosaccharide of lipid A-core is get attached by O-antigen and this is also caused by *waaL* gene (Y. Han et al., 2014). Additionally, this is revealed that *waaL* gene of APEC is involved in virulency through signature-mutagenesis (Li et al., 2005). Whether the *waaL* gene causes the APEC to be pathogen or not, this is yet to be found out.

In order to investigate the involvement of *waaL* gene in pathogenicity of APEC, either over expression of the target gene can be checked or a gene knock out stagey can be implemented. Gene knockout falls under the category of genome editing tools. These editing tools are competent means that give scientists an insight to edit the DNA of any organism by allowing genetic part to be edited by its removal or addition at a specific position in the organism's genome. Many applications for genome engineering have been acclaimed (Genetics Home Reference, 2018). Lately, scientists have hijacked the survival manoeuvre of prokaryotes. These are known as CRISPR/Cas and can edit the genome of any organism. It is basically natural defence system of bacteria by keeping portions of the invading viruses in their memory. Naturally, bacteria have an adaptive immune system kind of analogous to the way humans have antibodies. Nobody thought that bacteria i.e. single celled organism, would be capable of this kind of adaptation but it turned out that they are, and the scientists thought if they would hijack this innate immune system CRISPR system and could perhaps use it. Initially this technique was used for specific gene knockout in different organism but modifications in Cas enzymes have extended this to selectively

suppress or activate specific genes, to isolate specific portion of the DNA and to edit the RNA/DNA with great accuracy (Marraffini & Sontheimer, 2010).

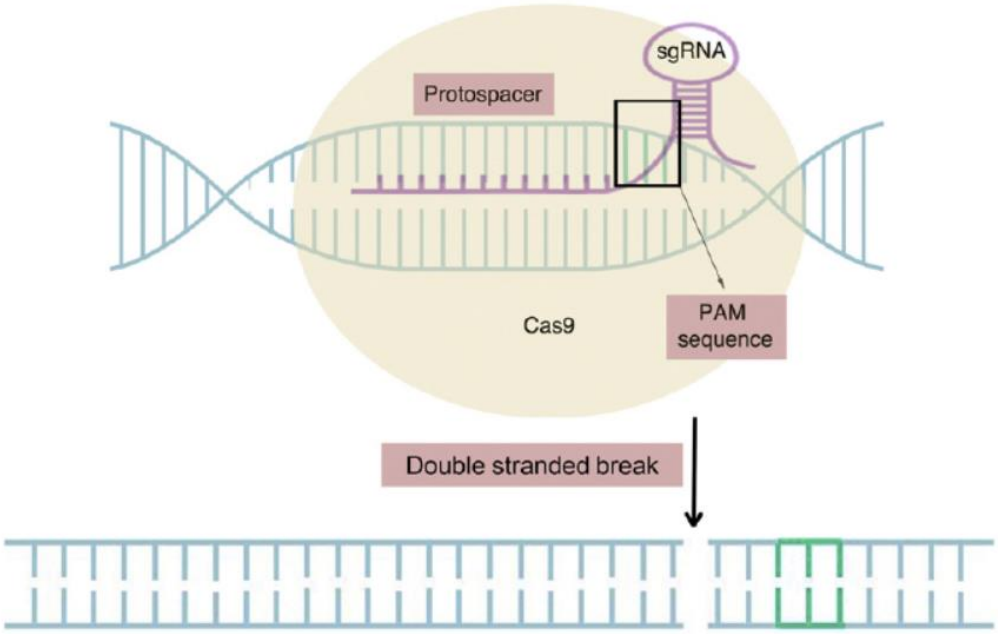

Figure 1.2 Edited genome form mediation by CRISPR/Cas9. Firstly, sgRNA recognize the sequencing of a target within the organism's genome. A DSB is made in a region, by nuclease of Cas9 that is close to sequence of PAM having NGG on 3' position (Ceasar et al., 2016).

Functional parts of CRISPR/Cas system include short repetitive sequence i.e. palindromic sequence separated by non-repetitive sequence known as spacers. In case of viral infection, part of invader's spacer duplicates and integrates inside the CRISPR locus and besides CRISPR locus, another locus embraces a pair of genes that encode the Cas (CRISPR Associated Endonucleases) proteins. This protein is involved in the breakdown of the targeted sequence of the invaders. As a result of repeated infection from same organism, invading RNA/DNA leads towards the formation of Cas and guide RNA (Crispr RNA and tracrRNA) against these exogenous molecules. Another vital part that provides basis for the genome editing is a short, unique single stranded (gRNA)

having a scaffold sequence which is basically a chimera (hybrid) of Crispr RNA (crRNA) and tracrRNA. Cas nuclease recognizes the Protospacer Adjust Motif (PAM) which should be present in gene of interest and cleaves the target sequence in editing of genome by CRISPR/Cas9, a sequence of target is recognized by gRNA inside genome with the help of complimenting pairing of the base. A DSB (double stranded break) is made by the nuclease of Cas 9 after this on the base pair of position 3 with the help of the sequence of PAM that is Cas9 having NGG inside (Hille & Charpentier, 2016).

In this study, LPS synthesis gene *waaL* was targeted in APEC by employment of CRISPR/Cas9 gene editing technique since the recognition of LPS as *E. coli*'s factor for virulence in a major way and has main functions in the bacterial pathogenesis with APEC (X. Han et al., 2013). CRISPR/Cas9 was utilized to generate knockout in the bacterial genome at specific site. CRISPR introduce DSB in genomic segments at points where Cas9 protein recognizes the PAM sequence. After DSB is introduced in the DNA strand, the cellular repair pathway mechanism gets activated and tries get repaired by NHEJ (Non-Homologous End Joining) nevertheless this restoration will most likely to be error prone that will result in insertions or deletions at target site. These in-dels often result in frame shifting thus resulting in inactivity of whole locus. Effective knockout of the *waaL* gene of APEC is expected by using this technology. It is expected that effective knockout of gene of interest will aid in understanding the role of *waaL* gene in pathogenicity of APEC.

2. Literature Review

2.1. *Escherichia coli* (*E. coli*)

As mentioned earlier that *E. coli* belongs to Enterobacteriaceae family that Theodor Escherich described in 1885 for the first time is commonly present in the food, environment and gastrointestinal tract of humans and animals (Van Elsas et al., 2011). *E. coli* has shapes of rods referred as coliforms and is gram negative. Coliforms generally reside inside the intestine of mammals where they play vital role in maintenance of healthy microbiota by fighting pathogens. *E. coli* specifically lives in the large intestine in mutually benefit relationship hence known as commensal bacteria. Still, *E. coli*'s strains that are pathogenic also exist and may cause serious fatal infections. Based on the highly adaptive nature of *E. coli*, some strains have acquired virulence properties through transposons, pathogenicity islands and plasmids *E. coli* become pathogenic (Tenaillon, Skurnik, Picard, & Denamur, 2010).

2.2. Pathogenicity of *E. coli*

DEC or Diarrheagenic *E. coli* and ExPEC or Extraintestinal pathogenic *E. coli* are known to be Pathogenic (Croxen & Finlay, 2010). ExPEC is normally found in the intestinal microflora but does not colonize the tissue but by breaching the intestinal epithelium barrier it can colonize other organs and can cause urinary tract infections (UTI) in humans, septicaemia and meningitis in new-born babies and colibacillosis in birds (Clermont, Christenson, Denamur, & Gordon, 2013) hence causing a high economic burden both for poultry industry and in terms of human health care costs and loss of productivity (Pitout, 2012). Another aspect of ExPEC epidemiology is the probability of it getting transferred in human bodies by the means food chain. This is revealed in a study, during preparation of raw poultry meat for cooking, bacteria which are present on the surface of

chicken or inside its organs can colonize the host GI and be excreted for up to ten days after preparation (Linton et al., 1977). Thus, APEC is not just a concern for poultry sector but it's also significant from public health point of view.

2.3. APEC; an etiologic agent of colibacillosis in poultry

APEC, the sub pathotype of ExPEC is the causative agent of colibacillosis and an important pathogen of the poultry industry thus resulting in significant diseases, mortality and subsequent economic losses and the determinant conditions linked with colibacillosis include cellulitis, coli-granuloma, omphalitis, swollen head syndrome, enteritis, septicaemia resulting in the death of the chicken in severe cases. The genome of *E. coli* holds diversity to a greater extent as it constitutes housekeeping genes that are important for basic cellular functionality and maintenance, resistome that have antibiotic resistance genes, pathogenicity islands etc. (Raimondi, Righini, Candeliere, Musmeci, Bonvicini, Gentilomi, Starčič Erjavec, Amaretti, & Rossi, 2019) and there are still number of genes whose pathogenicity needs to be investigated and the reasons of higher pathogenic behaviour of gram-negative bacteria than the bacteria having gram-positive properties lies in this fact; that the membrane that is outside the gram-negative bacteria has been made up of an endotoxin i.e. LPS and it has been considered important for gram-negative bacteria survival (Alexander & Rietschel, 2001).

2.4. Lipopolysaccharide Synthesis Gene *waaL*:

The most essential component that provides structural stability to the outer membrane of gram - negative bacteria is LPS, which is majorly comprised of three definite parts; O-antigen, lipid A and core oligosaccharide. Amongst these O-antigen plays a significant part in stimulating the inbuilt system of the host at the time of infection (Montminy et al., 2006). The product of the target

gene is WaaL protein and it plays an important role in joining of already mustered antigen to lipid A-core thus playing an important part in the synthetic pathway of LPS since the ligation of O-antigen to the surface of LPS is considered amongst the crucial steps (Y. Han, Han, Wang, Meng, Zhang, Ding, & Yu, 2014). Number of genes are found in synthesis of O-antigen including the O-antigen flippase (wzy), the lipid A-core ligase (*waaL*), the O-antigen transporter (wzx), and the regulator of O-antigen chain length (wzz) that plays an important role in the transport of O-antigen, which altogether maintain the function and structural stability of LPS O-antigen (Wang & Quinn, 2010). Besides all this information role of *waaL* gene in pathogenicity of APEC still needs investigation.

2.5. Genome editing technique; CRISPR/Cas9

CRISPR is an acronym for clustered, regularly interspaced, short palindromic repeat and Cas9 is CRISPR associated protein and this system display a prokaryotic adaptive immune system and it has taken place as the most popular tool in the field of genome editing and the reason why this technique has taken over the world of genome editing in recent years is because of its capability of Cas9 protein to induce double-stranded breaks (DSB) in the DNA strand by being cost effective and requires minimal effort unlike transcription-activator like effector nucleases (TALEN) and zine-finger nucleases (ZFN) (Ceasar, Rajan, Prykhozhij, Berman, & Ignacimuthu, 2016). This technique has been deployed in many species ranging from prokaryotes to humans as this technique promises to resolve various molecular biology problems in scientific research.

2.6. Components and mechanism of CRISPR/Cas9

CRISPR/Cas9 is present as an acquired innate system in prokaryotes and is considered as a defence system against plasmids and phages and this system contains;

- CRISPR Locus: CRISPR region comprises of short recurring sequence i.e. palindromic sequence which is separated by non-repetitive sequence known as spacers.
- CRISPR Associated Protein Cas9: along with CRISPR locus, another locus has pair of genes that encode for CRISPR associated endonucleases proteins, Cas9 which acts as a scissors and induces DSB in the DNA sequence.
- Guide RNA: gRNA is formed by the fusion of CRISPR RNA (crRNA) and Trans activating CRISPR RNA (tracrRNA).
- PAM Sequence: PAM is recognized by gRNA present in the target sequence of the genome via complementary base pairing (Ceasar, Rajan, Prykhozhij, Berman, & Ignacimuthu, 2016).

The genome editing process has two basic steps: DNA cleavage followed by DNA repair mechanism. The initial part of gene modification is exact generation of double stranded breaks (DSB) in the genome when gRNA recognizes the target sequence in the genome of the host organism and the Cas9 precisely makes a DSB at region near to PAM sequence which is NGG for Cas9 at its position 3'. After the generation of DSB, DNA repair mechanism is triggered by homology directed repair (HDR) or non-homologous end joining (NHEJ). As result of NHEJ at site of DSB thus resulting in knockout of gene either by mutation in the reading frame of target or protein part that is coded by that gene (Sander & Joung, 2014). The CRISPR/Cas9 technique has emerged as the most efficient and appealing tool for genome editing and it has found different applications in targeting various genes in many organism including bacteria, zebrafish, rabbits, mice, monkeys, humans etc (Ceasar, Rajan, Prykhozhij, Berman, & Ignacimuthu, 2016).

3. Materials and Methods

3.1. Materials

This part of materials consists of all the softwares, online tools, equipment, enzymes, chemicals, oligonucleotides, plasmids, bacterial strains, buffers and media used during the research work.

3.1.1. Online tools and software used

Table 3.1 List of all online tools and softwares used in this study

Name	**Function**	**Link**
NCBI	Sequence Retrieval, Blast	https://www.ncbi.nlm.nih.gov/
JackLin's CRISPR/CAS9 gRNA Finder	Designing of CRISPR/Cas guide RNA with reduced off-target sites	Jack Lin's CRISPR/CAS9 gRNA Finder
NEBcutter V2.0	Finds restriction sites in your plasmids/DNA sequences	http://nc2.neb.com/NEBcutter2/
SnapGene	Fast and easy to propose, foresee DNA cloning	https://www.snapgene.com/

Primer3 Input (version 0.4.0)	Designing of PCR primers	http://bioinfo.ut.ee/primer3-0.4.0/
Tm Calculator (Thermo Fisher scientific)	Allows to figure out melting and annealing temperature of primers	https://www.thermofisher.com/pk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html

3.1.2. Equipment used in this study

Table 3.2 Equipment used during this study

Equipment	**Manufacturer**
2720 Thermal Cycler	Applied Biosystems
Heat Block	Wealtec corp.
Nanodrop	Colibri
Concentrator Plus	Eppendorf
Shaking Incubator	Jsr
Gel Electrophoresis Tank	Cleaver Scientific Ltd
Ultraviolet Viewing cabinet	Extra Gene
Shaking Water Bath	Memmert
Scanfrost	Caravell
Tabletop Balance	ShiMADZu
Hot Plate	Velp-Scientifica

Centrifuge Machine	Hermle
pH Meter	WTW inoLab
Microwave Oven	Haier
Laminar Flow cabinet	Esco
Microcentrifuge	Sigma
Vortex Mixer	Heidolph
Spectrophotometer	Optima
Incubator	Memmert
Electroporator	Eppendorf
Gel Dolphin Doc	Weal Tech ELITE 300 Plus
Real Time PCR	Applied Biosystems 7300s
Monarch DNA Gel Extraction Kit	New England Biolabs

3.1.3. Chemicals

Table 3.3 Chemicals used during this study

Chemicals	**Manufacturer**
Agar	Bioworld USA
Agarose	Bioworld USA
Nutrient Broth	Lab M UK
Tryptone	Bioworld USA
Nuclease Free Water	Caisson labs
Crystal Violet	Daejung
Yeast Extract	Lab M UK

Methanol	Chem Lab
Ethanol Absolute	Sigma Aldrich
Ethidium bromide	Sigma Aldrich
Eosin methylene blue agar	Lab M UK
Tryptone soya agar	Lab M UK
Sodium Chloride	BDH Laboratories

3.1.4. Antibiotics used in this study

Table 3.4 Antibiotics used during this study

Antibiotics	**Symbol**	**Stock solution (mg/ml)**	**Conc. used for *E. coli* (µg/ml)**	**Dissolution media**	**Manufacturer**
Chloramphenicol	chl	17 mg/ml	25µg/ml	75% EtOH	ACTGene
Kanamycin	kana	10 mg/ml	34µg/ml	water	Phytotechlab

3.1.5. Molecular biology grade Enzymes used

Table 3.5 List of Molecular biology grade Enzymes used

Enzymes	**Manufacturer**
RNase A	Thermo scientific
T4-Polynucleotid-Kinase (PNK)	Thermo scientific
T4-DNA-Ligase	Thermo scientific
Eco31I (*BsaI*)	Thermo scientific
Taq-DNA-Polymerase	Thermo scientific

3.1.6. Molecular biology grade Markers used

Table 3.6 List of Molecular biology Markers used

Marker	**Manufacturer**
GeneRuler 1kb DNA Ladder	Thermo scientific
GeneRuler 100bp DNA Ladder	Thermo scientific
GeneRuler 50bp DNA Ladder	Thermo scientific

3.1.7. Bacterial strains used

Table 3.7 List of Bacterial strains used

Bacteria	**Description**	**Source/Reference**
Avian pathogenic *Escherichia coli* (APEC O1)	Causative agent of colibacillosis	Microbiology Lab, University of Agriculture Faisalabad
DH5α	Helper strain of *E. coli* used for transformation	AntiBacter Research Lab, ASAB, NUST

3.1.8. Plasmids used

Table 3.8 List of Plasmids used

Plasmids	**Description**	**Source/Reference**
pCRISPR	A crRNA expression plasmid used for targeting a specific sequence.	Luciano Marraffini (Addgene plasmid # 42875)
pCas9	Bacterial expression of Cas9 nuclease, tracrRNA and crRNA guide	Luciano Marraffini (Addgene plasmid # 42876)

3.1.9. Oligonucleotides used during this study

Table 3.9 Oligonucleotides used during this study

Oligonucleotides	Sequence	Reference
waaL-gRNA-1	5' AAACTTGGGCCACGATATGCATTG 3'	This study
waaL-gRNA-2	5' AAAACAATGCATATCGTGGCCCAA 3'	This study

3.1.10.Microbiological Media

Distilled water was used to prepare all the required solutions, buffers and media. The media was prepared and autoclaved in order to maintain the sterility. Autoclaving was carried out at 121ºC for 15 minutes. pH was adjusted at 7.0 unless otherwise mentioned.

Table 3.10 Nutrient Broth (N Broth)

Sr. No	Components	Quantity (g/L)
1	Peptone	5.0
2	Beef Extract	3.0

Table 3.11 Nutrient Agar (N Agar)

Sr. No	Components	Quantity (g/L)
1	Peptone	5.0
2	Beef Extract	3.0
3	Agar	15.0

3.1.11.Media for Motility Assays

Table 3.12 Swimming Motility Media

Sr. No	Components	Quantity (g/100ml)

1	Tryptone	1.0
2	NaCl	0.5
3	Agar	0.3

Table 3.13 Swarming Motility Media

Sr. No	Components	Quantity (g/100ml)
1	Nutrient broth	0.8
2	NaCl	2.0
3	Agar	0.5

Table 3.14 Twitching Motility Media

Sr. No	Components	Quantity (g/L)
1	Tryptone	10.0
2	NaCl	10.0
3	Agar	10.0
4	Yeast extract	5.0

Table 3.15 1% Crystal Violet Solution

Sr. No	Components	Quantity (g/100ml)
1	Crystal violet	1.0
2	Distilled water	100.0

3.1.12.Solutions for Microtiter Assay

Table 3.16 0.1% Crystal Violet Solution

Sr. No	Components	Quantity (g/100ml)

1	Crystal violet	0.1
2	Distilled water	100.0

Table 3.17 0.85% Sodium Chloride

Sr. No	Components	Quantity (g/100ml)
1	NaCl	0.85
2	Distilled water	100.0

Table 3.18 33% Glacial Acetic Acid

Sr. No	Components	Quantity $100ml^{-1}$ (v/v)
1	Glacial acetic acid	33.0
2	Distilled water	77.0

3.1.13. Solutions used in Plasmid extraction

Solution I (Resuspension buffer)

Table 3.19 Solution I (resuspension buffer)

Sr. No	Components	Quantity
1	Glucose	0.05 M
2	Tris/HCl	0.025 M
3	EDTA	0.01 M (pH 7.4)

Solution II (Digestion/lysis buffer)

Table 3.20 Solution II (digestion/lysis buffer)

Sr. No	Components	Quantity
1	NaOH	0.2 N
2	SDS	1 %

Solution III (precipitation buffer)

Table 3.21 : Solution III (precipitation buffer)

Sr. No	Components	Quantity
1	KAc	5 M (pH 5.6)
2	Acetic acid	2 M

3.1.14.Buffer used for Gel Electrophoresis TAE (1x)

Table 3.22 TAE (1x) Buffer

Sr. No	Components	Quantity
1	Tris-HCl	40 mM
2	Acetic acid	40 mM
3	EDTA	0.4 mM (pH 8.0)

3.1.15.Solutions for competent cells production

Table 3.23 Composition of solutions for competent cells preparation

Solution	Components
Solution 1 (autoclaved)	• $MgCl_2$ (0.5M) • $CaCl_2$ (0.5M)

	• ddH_2O
Solution 2 (autoclaved)	• $CaCl_2$ (0.5M) • Glycerol 50% • ddH_2O

3.1.16. Antibiotics used for Antibiotic Susceptibility Testing:

Table 3.24 Antibiotics used in the study

S. No.	Antibiotic	Abbr.	Category	Conc. (µg)	S	I	R
1.	Amoxicillin	AML	Penicillin	10	≥ 17	14-16	≤ 13
2.	Ampicillin	AMP	Penicillin	10	≥ 17	14-16	≤ 13
3.	Amoxicillin-clavulanate	AMC	β-lactam combination agent	20/10	≥ 18	14-17	≤ 13
4.	Doxycycline	DO	Tetracycline	30	≥ 14	11-13	≤ 10
5.	Tetracycline	TE	Tetracycline	30	≥ 15	12-14	≤ 11
6.	Piperacillin-tazobactam	TZP	Penicillin- β-lactamase inhibitor	100/10	≥ 21	18-20	≤ 17
7.	Eratapenem	ETP	Carbapenem	10	≥ 22	19-21	≤ 18
8.	Meropenem	MEM	Carbapenem	10	≥ 23	20-22	≤ 19
9.	Imipenem	IPM	Carbapenem	10	≥ 23	20-22	≤ 19
10.	Gentamicin	CN	Aminoglycoside	10	≥ 15	13-14	≤ 12
11.	Tobramycin	TOB	Aminoglycoside	10	≥ 15	13-14	≤ 12

12.	Streptomycin	S	Aminoglycoside	10	≥ 15	12-14	≤ 11
13.	Amikacin	AK	Aminoglycoside	30	≥ 17	15-16	≤ 14
14.	Ciprofloxacin	CIP	Fluoroquinolone	05	≥ 21	16-20	≤ 15
15.	Norfloxacin	NOR	Fluoroquinolone	10	≥ 17	13-16	≤ 12
16.	Levofloxacin	LEV	Fluoroquinolone	05	≥ 17	14-16	≤ 13
17.	Trimethoprim-sulfamethoxazole	SXT	Folate Pathway Antagonist	25	≥ 16	11-15	≤ 10
18.	Nitrofurantoin	F	Nitrofuran	300	≥ 17	15-16	≤ 14
19.	Erythromycin	E	Macrolide	15	≥22	17-21	≤ 18
20.	Ceftriaxone	CRO	Cephalosporin	30	≥ 23	20-22	≤ 19
21.	Cefotaxime	CTX	Cephalosporin	30	≥ 26	23-25	≤ 22
22.	Ceftazidime	CAZ	Cephalosporin	30	≥ 21	18-20	≤ 17

* Abbr. = Abbreviation Conc. = Concentration

S = Sensitive I = Intermediate sensitivity R = Resistant

The table describes the antibiotics used in the study including the categories and zone sizes for interpretation of susceptibility or resistance of bacterial isolates (CLSI 2018).

3.2. Methodology for In-silico Analysis

3.2.1. Sequence retrieval of *waaL* gene:

Sequence of *waaL* gene of APEC O1 was retrieved from NCBI in FASTA format.

3.2.2. gRNA/oligonucleotide design:

In order to design gRNA, Jack Lin's CRISPR/CAS9 gRNA Finder software was used. For that purpose, input sequence of *waaL* gene was added. For designing gRNA some points were kept in mind;

- **GC content:** The GC content should range in between 40%-80%
- **Length:** The length should lie in between 17-24 base pair
- **PAM sequence:** Target must be next to protospacer adjacent motif
- **Target sequence specificity:** The target sequence should be specific in the whole genome to avoid off-target editing.

Table 3.25 Sequence of oligonucleotides

Sr. No	Name	Sequence
1	waaL gRNA 1	5' AAACTTGGGCCACGATATGCATTG 3'
2	waaL gRNA 2	5' AAAACAATGCATATCGTGGCCCAA 3

3.2.3. Selection of Vector Plasmids (Expression Cassettes):

For transformation and expression of the CRISPR and CAS9 nuclease into the bacterial cells, vector is required. For that purpose, plasmid vectors pCRISPR and pCas9 were ordered from Addgene. pCRISPR vector was used for the successful expression of gRNA sequence into the host whereas pCas9 was utilized for the expression of CAS9 nuclease protein inside the bacterial cells.

3.3. Wet Lab Methodology:

Plasmids were provided in the form of agar stab from Addgene and vectors, pCRISPR and pCas9 were streaked on nutrient agar plates having Kanamycin and Chloramphenicol antibiotics in order

to get single colonies. Agar plates were kept at 4ºC and primary culture were grown overnight in incubator at 37ºC.

3.3.1. Stock Preparation of Plasmids and storage:

Plasmids were taken from the vials with the aid of inoculation loop and were added to the already labelled conical flasks having Kanamycin for pCRISPR and Chloramphenicol for pCas9 plasmids. The flasks contained Luria Broth (LB) media. After inoculation under sterile conditions the flasks were kept overnight in the shaking incubator at 37°C. Next day, the flasks were taken out of the incubator and 1.5 ml of overnight culture was taken in already autoclaved eppendorf tube and was centrifuged for 5 min at 5000 rpm. The supernatant was discarded, and the step was repeated once or twice in order to obtain pellet. After that the supernatant was again discarded and the pellet was resuspended in 1ml LB. Suspension having plasmids and LB were then added to already labelled cryovials having 500µl of 80% glycerol. Vortex was used in order to thoroughly mix the glycerol and the culture having cells. Liquid nitrogen was used to freeze the samples and were stored at -80°C and -20°C for downstream processing.

3.3.2. Plasmid Isolation (Miniprep):

Plasmid DNA was isolated from the cells based on alkaline lysis principle in small culture volume hence known as miniprep. Overnight grown cell culture was taken in autoclaved eppendorf and was centrifuged at 5000 rpm for 5 min. After centrifugation the supernatant was discarded. More culture was added again if the pellet was not enough. The culture pellet was obtained after discarding the supernatant. To that pellet 200 µl ice cold solution-I (Table 3.19) was added. RNase A (4 µl /ml) was added fresh to solution-I every time. It was then incubated on bench for 5 min on bench. Then solution II or lysis buffer (Table 3.20) was added and the eppendorf was inverted several times for the lysis of cells. For the precipitation of chromosomal DNA of the cells 200 µl

of ice-cold solution III (Table 3.21) was added and incubated on ice for 15 min. Chromosomal DNA was visualized as filamentous, white precipitate. The mixture was then centrifuged in refrigerated centrifuge machine at 13,000 rpm for 15 min at 4°C. Chromosomal DNA and cell debris got sedimented after centrifugation while the plasmid DNA remained in the supernatant. The supernatant containing the plasmid DNA was transferred carefully into a new autoclaved eppendorf and 500 µl of chilled isopropanol was added to precipitate the plasmid DNA by centrifugation at 13,0000 rpm at 4°C for 15 min. After centrifugation pellet was washed with 100 µl of 80% ethanol and again centrifuged at 13,000 rpm for 10 min at 4°C.Pellet was dried in Speed Vac to remove any left-over ethanol if present and then dissolved in 50 µl ddH_2O.

3.3.3. Agarose gel electrophoresis:

For agarose gel electrophoresis, 1% gel was prepared by dissolving 0.5 grams of agarose in 50 ml of 1X TAE buffer (Table 3.22) by boiling. After complete dissolution the gel was cooled down for few minutes and then ethidium bromide was added for stain. The gel was poured into a taped gel casting tray which already had comb. After polymerization, the gel was transferred in the gel tank and the tank was filled with 1X TAE buffer. Samples were mixed with 6x DNA loading dye. 1kb, 100bp, 50bp ladders were used for reference. Electrophoresis was carried at 80V for 45-50 min.

3.3.4. Oligonucleotide Annealing and Phosphorylation:

Oligonucleotides were annealed by heat block method. Forward and reverse were annealed together by making a 50 µl reaction volume comprised of 22 µl of each waaL-gRNA-1 and waaL-gRNA-2 oligos listed in (Table 3.28) 5 µl of 10X T4 ligase buffer was added along with 1 µl of T4 PNK (T4 Polynucleotide Kinase) which enabled 5' phosphorylation of DNA for ligation and it also removed 3' phosphoryl groups. For annealing, 2.5 µl of 1M NaCl was added. It was incubated at 95°C in a thermocycler followed by placing the samples at room temperature for 2 hours.

Annealed oligos were diluted 10 times and prior to their storage at 4°C they were loaded on agarose gel for the confirmation of hetero-dimer formation.

3.3.5. Restriction Digestion of Vectors:

Restriction digestion of pCRISPR was done with BsaI enzyme. For that purpose, 2 µl of 10x buffer G was added, plasmid having concentration of (0.5-1 µg/µL) was added in quantity of 1 µl. Restriction enzyme Eco31| (*BsaI*) was added in between the range of (0.5-2 µl) and 16 µl of NF water was added to make 20 µl reaction volume. After adding all the constituents, it was gently mixed in vortex for few seconds and were placed in the incubator for up to 16 hours at 37°C. for confirmation of successful digestion samples were loaded in agarose gel and visualized by UV illuminator.

3.3.6. Gel Purification of digested Vector:

Prior to ligation, gel purification of digested pCRISPR was done which is also recommended for successful cloning. For that purpose, the digested vector sample was loaded in a larger well in electrophoresis tank. The band from the gel having digested vector was cut out with the help of sterile blade/razor and was transferred to 1.5 ml microcentrifuge tube. The microcentrifuge tube was measured first when it was empty then it was measured with the gel mass and the gel weigh was subtracted from the microcentrifuge tube weigh. The gel weigh was around 680mg, so 2.72 ml of dissolving buffer was added to the gel slice according to the protocol that stated to add 4 volumes of gel dissolving buffer to gel slice for instance, for 400 µl buffer per 100 µl or 100 mg agarose. The gel sample was incubated, and they were vortexed from time to time until the gel was completely dissolved. Upon complete dissolution sample was loaded onto the column, the cap was closed, and it was spun for 1 min and the flow through was discarded. The column was transferred into a collection tube and 200 µl of DNA wash buffer was added and again centrifugation for 1

min was done and this process was repeated once again to ensure any leftover flow through. The column was transferred to a clean microcentrifuge tube and 6 µl of elution buffer was added to the center of the matrix and after incubation for 1 min centrifugation was done to elute DNA.

3.3.7. n-Butanol Precipitation:

For removal of different salt contaminations or other impurities from DNA/plasmids which can cause obstruction in transformation or PCR reactions, samples were precipitated by n-Butanol. Sample needed to be purified was first diluted with 50 µl ddH_2O. After that it was mixed with 500 µl n-Butanol. The mixture was shaken vigorously and centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was discarded, and the pellet was washed with 80% ethanol and again centrifuged for 10 min. Resulting pellet was air dried in Speed Vac and for further use it was resuspended in 20 µl of ddH_2O.

3.3.8. Ligation of gel purified pCRISPR and gRNA:

In order to get a plasmid having gRNA sequence ligation was done of already digested and gel purified pCRISPR and hetero dimer. Ligation calculator was used to calculate the concentration of vector and insert. Prior to these calculations' nano drop was used and the concentrations were adjusted by addition of nuclease free water. Mostly insert to vector ratio is 1:3. So 2 µl of annealed oligonucleotides along with 6 µl of vector (pCRISPR) was added to which 2 µl of T4 DNA ligase and 2 µl of 10X T4 ligase buffer was added. Reaction volume was raised to 20 µl by addition of 8 µl of nuclease free water. The ligation mixture was kept at 16°C for overnight ligation. Ligases were deactivated at 65°C for 10 min before transformation for better results and store at 4°C for future use.

3.3.9. Competent cell preparation of DH5α:

Competent cells were prepared according to the method described by (Chang et al., 2017). DH5α, cells from cryo vials were taken and streaked on nutrient agar plates and incubated overnight at 37°C to get isolated colonies. Next day, isolated colonies from the petri plate were taken and were added to the conical flasks containing nutrient broth. Flasks were incubated overnight in a shaking incubator at 37°C by keeping speed of incubator at 200 rpm in order to get growth. Next day after incubation, 1 ml of overnight or mother culture was taken and was subcultured in another flask having 50 ml of fresh nutrient broth. This culture was again incubated for an hour to two till its optical density OD_{600} reached 0.5 to 0.6. The culture was transferred to already ice- chilled 50 ml falcons and was placed on ice for 20 min. After incubation on ice, falcons were centrifuged at 4°C at 4000 rpm for 10 min. The supernatant was discarded, and pellet was resuspended in 20mL ice-cold 0.1M $CaCl_2$ and incubated on ice for 30 min. Again, centrifugation was done at 4°C at 4000 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in 5ml ice-cold 0.1M $CaCl_2$. The resuspended pellet was aliquoted as 50µl in already chilled microcentrifuge tubes and liquid N_2 was used for snap freezing purpose. These competent cells were immediately used for transformation and rest of the cells were stored at -80°C with 15% glycerol.

3.3.10. Transformation of pCRISPR+gRNA in DH5α by Heat Shock Method:

In order to transform ligated vector i.e. pCRISPR having insert i.e. gRNA into the competent cells of DH5α heat-shock method was used (Chang, Chau, Landas, & Pang, 2017). Competent cells were thawed on ice. The ligated plasmid 1-5µl (10pg-100ng) was added to 50µl cell aliquot of competent cells. The sample was incubated on ice for 30 min. After incubation on ice the sample was placed in 42ºC water bath for 30 seconds. The cells were placed on ice for 2 min, to which 1ml pre-warmed LB medium was added and incubated in shaking incubator at 37ºC, 200 rpm, 1

hour for outgrowth. 100µl transformation mixture was spread on the plates having kanamycin as an antibiotic selection marker and the plates were incubated at 37ºC for 12-16 hours.

3.3.11. Confirmation of Single Transformation by Colony PCR:

For the verification of successful ligation of pCRISPR having gRNA colony PCR was done and for that purpose the forward primer was designed in the region of vector i.e. pCRISPR and reverse primer was taken from the gRNA.

Table 3.26 Primers of colony PCR

Sr. No	Name	Sequence
1	Forward Primer	5' AGTCGTGTCTTACCGGGTTG 3'
2	Reverse Primer	5' AAAACAATGCATATCGTGGCCCAA 3'

- **Steps of Colony PCR:**

For Colony PCR, 20µl of NF water was taken in PCR tubes and transformed colonies from the antibiotic plates were picked carefully and mixed with NF water in the PCR tubes until the solution got turbid. The tubes were spun in the mini spin. After spinning, the PCR tubes were placed in the thermocycler for 10min at 95ºC followed by refrigerated centrifugation at 4ºC, 6000 rpm for 3 min. PCR mix was prepared having reaction volume of 25µl which comprised of 12.5µl of master mix, 1µl of forward primer and 1µl of reverse primer along with 2µl of PCR water having transformed colonies and NF water was added to complete the volume. PCR was performed using the profile i.e. 95ºC for 5 min, succeeded by 30 cycles at 95ºC for 30 seconds, 57ºC for 45 seconds and 72ºC for 40 seconds finally followed by extension cycle at 72ºC for 10 min. Successful verification of amplification via colony PCR was assessed by gel electrophoresis.

3.3.12. Electrocompetent cell preparation of APEC O1:

In order to prepare electrocompetent cells of APEC O1, cells from cryovials were streaked on agar plates and were incubated overnight at 37°C to get isolated colonies. Next day, isolated colonies from the petri plate were taken and were added to the flasks containing liquid broth. Flasks were incubated overnight in a shaking incubator at 37°C by keeping the speed of incubator at 200 rpm in order to get growth. Following day, after 10 ml of mother culture was taken and was subcultured in another flask having 500 ml of fresh broth. This culture was again incubated for two hours until its optical density OD_{600} reached 0.4- 0.6. The culture with the desired OD was transferred to already chilled 50ml falcons. Refrigerated centrifugation was done at 4ºC, 8500 rpm for 10 min. The pellet was washed with autoclaved distilled water thrice by centrifugation followed by resuspension. DMSO/ Glycerol was added for storage at -80ºC for downstream processing.

3.3.13. Co-transformation of pCRISPR+gRNA and pCAS9 in APEC O1:

As a result of co-transformation, the expression of pCRISPR will enable the guide RNA to be expressed whereas pCAS9 will aid in the expression of CAS9 protein which will eventually lead to cut on the target site because of its nuclease activity. In order to transform both plasmids via electroporation, electrocompetent cells were taken from -80ºC freezer and thawed on ice. Electroporation cuvettes were placed on ice along with samples. Plasmids (3-5µl) were added to 50µl aliquots of electrocompetent cells of APEC O1. After the addition of both plasmids into the competent cells they were mixed gently on ice. That mixture was added to the ice-chilled cuvettes and the excess moisture outside of the cuvette was removed using a tissue wipe and the cuvette was then place inside the electroporator. The lid was closed and an electric pulse of 1800V for 3.6 milliseconds the plasmid DNA passed into the cells. The cuvette was removed from the chamber after the electric shock and 1ml of LB was added immediately to the transformed cells and

incubated at 37ºC for 1 hour in shaking incubator. Later, the transformation mix was spread on the agar plates having both antibiotics that are kanamycin and chloramphenicol, the plates were incubated overnight at 37ºC.

3.3.14. Colony PCR for the confirmation of Co-transformed DH5α:

The results of co-transformation were observed in the form of colonies on the antibiotic plates the very next day after electroporation. In order to further verify among the false clones and the transformed clones, the colonies were picked from the plates and were subjected to colony PCR as mentioned in section 3.3.11. One set of primers were designed inside the gRNA and the other primer was designed in the downstream region of the gene. The PCR product was further sent for sequencing purpose.

The mutants having gene knockout were designated as M1-O1, M12-O1 and wild type of APEC O1 was named as WT-O1 was further exposed to several tests for their evaluation.

3.4. Growth Curve:

Mostly commonly used method in order to evaluate the growth phase of the bacterial population is the growth of a microbe in liquid nutrient medium further followed by serial dilutions and plating on agar plates (Johnson & Case, 1992). Resulting colonies are attributed as a colony forming unit (CFU). In order to understand the growth of a microbe, nutrient broth (Table 3.10) was prepared and autoclaved accordingly. The media was inoculated with bacterial culture and incubated at 37ºC. The optical density $O.D_{600}$ was first adjusted at 0.01. After the adjustment of optical density, the media was again incubated at 37ºC and optical density was measured at 600nm after 0, 0.5, 1, 1.5, 2, 4, 6,8. 10,24, 27,48, 72 and 96 hours. Graph was plotted by taking $O.D_{600}$ at Y-axis and time (hr.) on X-axis.

3.5. Survival rate under physiological stress conditions:

Besides the growth rate of M1, M12 and WT-O1 strains were observed, these strains exposed to some physiological stress conditions as described in (X. Han, Bai, Liu, Dong, Liu, Song, Ding, Qi, Liu, & Yu, 2013).

3.5.1 Osmolarity Endurance

3.5.2 Heat Shock

3.5.3 Acid Endurance

3.5.4 Alkali Endurance

First the bacteria were grown in brain heart infusion (BHI) broth, incubated overnight at 37ºC in static incubator. The next day the bacterial cells were collected by centrifugation at 3000×g for 5 minutes. Number of cells were measured by adjusting their OD at 600 nm. The bacterial cells were resuspended and further diluted to 10^7 cells/mL in phosphate buffer saline (PBS) for following endurance tests

3.5.1. Osmolarity Endurance:

In order to check the survival rate of bacteria under high osmolarity condition, bacteria were mixed with almost an equal volume of 4.8M NaCl and incubated for 1 hour at 37ºC, followed by 10-fold serial dilution in 0.9% saline and plated on agar plates in duplicates with subsequent 12-hour incubation.

3.5.2. Heat Shock:

For heat shock, 100µl of bacterial cell suspension were given heat shock in water bath at 54ºC for 3 min. The heated samples were 10-fold serial diluted and each sample was counted by plating onto agar plates for 12-hour incubation.

3.5.3. Acid Endurance:

In order to check the acid endurance rate 100ml of Liquid broth whose pH was adjusted with CH_3COOH at 3.00 was added to 100µl of cell suspension and was incubated at 37ºC for 20 min. The treated samples were plated on agar plates by serial dilutions as explained in section 3.5.1.

3.5.4. Alkali Endurance:

For alkali endurance 800µl of Tris-HCL (pH: 10:00) and 100µl of distilled water was added into 100µl of cell suspension and incubated at 37ºC for 20 min further plated on agar plates as explained in 3.5.1 section.

3.6. Microtiter Assay for Biofilm quantification of Wild Type and Mutants of APEC 01:

Biofilm comprise of microbial communities which are attached to surfaces in medical, natural and industrial setups. Bacterial communities settle themselves as irrepressible so they can sustain in acute condition. The microtiter plate assay is an important tool for the study of the early stages in biofilm formation and considered as one of the reliable methods for the detection of biofilm (Stepanović et al., 2000). Biofilm forming ability of the WT-O1, M1-O1 and M12-O1 was observed with the help of 96 -well microtiter assay. In order to grow biofilm WT-O1, M1-O1, M12-O1 and *bacillus* which served as a control were grown overnight in Tryptic Soy Broth (TSB) in a shaking incubator at 37ºC. Optical density of the mother culture was adjusted at 1 at 600nm with the help of spectrophotometer. After adjusting the O.D the samples were vortexed and 20µl of the bacterial culture was transferred into the wells of the 96-well microtiter plate, along with 1980µl of broth. For every biofilm assay *bacillus spp.* was taken as a positive control while TSB media was used as a negative control. Plates were covered with sealing tape and incubated aerobically at 37ºC in static incubator for 1,3,5 and 7 days.

After incubation the bacterial culture was dumped out by turning the plate over and shaking out the liquid. The 96-well plate was washed with 0.85% saline (Table 3.17) in order to remove loosely attached cells of bacteria. Plate was air dried for 30 min. In order to fix the tightly bound cells into the wells of the plate 200µl of methanol was added to each well and left for 15 min after which methanol was discarded and the plate was again air dried. Tightly bound cells were then stained with 200µl of 0.1% crystal violet per well for 10 min. After staining, the stain was discarded, and the remaining stain was removed by washing each well thrice with 0.85% saline. For quantification of tightly bound cells, 200µl of 33% glacial acetic acid (Table 3.18) was added in each well. The 96-well plate was left for 15 min at room temperature. Absorbance was noted at 595nm in microtiter plate reader, for confirmation of results, each experiment was done in triplicates.

3.7. Growth of Wild Type and Mutants of APEC O1 in Human Urine:

Bacteriuria refers to the ability of bacteria to survive in urine since urine is antimicrobial and retards growth of bacteria. Hence bacteria which can survive and grow in high urea conditions and urine have been indicated as one of the causative agents of urinary tract infections (UTI). Since *E. coli* is apprehended as major causative agent of UTI in almost 80% of all UTI conditions (Ipe & Ulett, 2016). In order to check if WT-O1, M1-O1 and M12-O1 without *waaL* gene can survive in urine or not this test of growth in human urine was performed.

Prior to the test, urine samples were taken from two healthy females and males which had no history of recent UTI and have not been on any medication as well. The urine samples were taken, filter sterilized, pooled and poured as aliquots in 2ml eppendorf tubes and stored at -20ºC. WT-O1, M1-O1 and M12-O1 were inoculated in Tryptic soy broth (TSB) and incubated overnight at 37ºC. Next day OD of each bacterial culture was adjusted at 1:00. Each bacterial culture was inoculated in filter sterilized urine in 1:100 dilution and 200µl of each urine inoculated sample was

placed in 96-well microtiter plate. For negative control DH5-α was used and UPEC was used as positive control. The plate was incubated at 37ºC for 8 hours duration under static conditions. After the completion of incubation time OD was measured at 600nm by using Elisa plate reader. The experiment was repeated in duplicates in order to get accurate values.

3.8. Motility Assays of Wild type and Mutants of APEC O1:

Bacteria move in order to obtain nutrients with the help of flagella, Pilli or other means (Mattingly et al., 2018). Three types of motility assays were performed for WT-O1, M1-O1 and M12-O1

- Swimming Motility
- Swarming Motility
- Twitching Motility

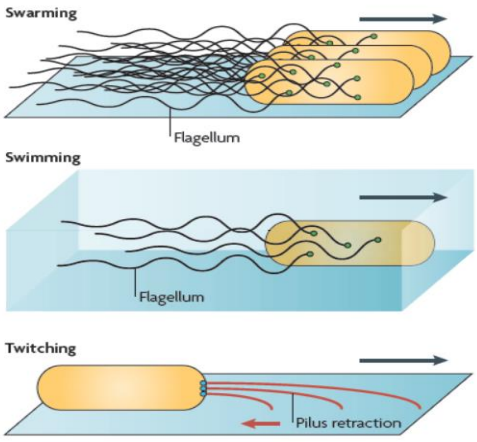

Figure 3.1 Bacterial Motility Mechanisms. Black arrows represent direction movement (Kearns, 2010)

3.8.1. Swimming Motility:

This motility is powered by rotating flagella and it's the movement of individual cell. For swimming motility assay tryptone swimming plate (Table 3.12) were prepared and left at room temperature for 6 hours followed by bacterial samples stabbed with sterile toothpick. The plates were left for incubation at 37ºC for 18 hours. After 24-hour, motility pattern was assessed by observing the circular turbid zone formed by the bacterial cells migrating from the actual point of incubation (Qiao et al., 2012).

3.8.2. Swarming Motility:

Swarming motility is referred to multicellular rotating flagella movement on semisolid media. In order to perform swarming motility assay 0.3% agar concentration is required to observe swarming motility however, agar concentration above this percentage obstruct swimming motility and support swarming motility on the other hand. While agar concentration of agar exceeding 1% inhibits swarming motility. The media was prepared for swarming motility as mentioned in (Table 3.13). Plates were kept at room temperature for 6 hours after the media was poured in them followed by stabbing method in which each bacterial colony was stabbed with sterile toothpick and plates were incubated at 37º C for 18 hours. Plates were assessed for swarming motility after 24-hour incubation.

3.8.3. Twitching Motility:

Twitching motility is powered by retracting movement of type IV pili, due to which bacteria are capable to move on solid or semisolid medium. Twitching motility basically helps in colonization of host and it happens via attachment, extension and then retraction of polar type pili IV. For twitching motility, plates were prepared according to the method described by Meng *et al*., 2005 (Table 3.14) and around 3ml twitching media was poured in the plates and left for drying at room

temperature for 6 hours followed by stabbing with bacterial cultures with sterile toothpick and left in incubator at 37ºC.After incubation the twitching media was discarded and washed. Methanol was used in order to fix the attached cells and further stained with 1% crystal solution for 15 min. Plates were washed after staining and the stained zone size was measured afterwards.

3.9. Antibiotic Susceptibility testing of the Wild Type and Mutants of APEC 01:

WT-O1, M1-O1 and M12-O1 were further assessed for Antibiotic susceptibility as reported by the instructions of European Committee on Antimicrobial Susceptibility (EUCAST). The antibiotics used in this testing are listed in (Table 3.27). The cultures were grown overnight in agar plates. The next day, each colony was mixed with saline and the turbidity was adjusted in comparison with 0.5 McFarland standard solution. In order to make uniform bacterial lawns 100µl of bacterial cell suspensions were used on MH agar plates followed by placing antibiotic discs on the bacterial lawn. Plates were then incubated at 37º for almost 16-18 hours after which zone of inhibition of antibiotics was noted.

4. Results

4.1. *In Silico* gRNA Designing:

4.1.1. Sequence Retrieval of *waaL* Gene:

Sequence of the target gene i.e. *waaL* was retrieved in FASTA format from NCBI (Fig 4.1). The length of *waaL* gene is 1254 bp. The *waaL* gene is involved in encoding O-antigen ligase, responsible for attachment of O-antigen ligase to lipid A-core oligosaccharide (Han *et al*., 2014). It is also involved in LPS synthesis that has been reported as an important virulence factor in *E. coli* (Y. Han, Han, Wang, Meng, Zhang, Ding, & Yu, 2014).

```
>CP000468.1:4090318-4091571 Escherichia coli APEC O1, complete genome
TTACTTATCTAATAAACATTGGTCCGATTGTACTTTAAAATAAGCACAAAGCAATATTATGGTGACAAAC
ACAGCAGTCGGAACCGGTTTAGAAAAGAGTATTACATCACTTAGGCCTACTACCAGTAGTGAAAAAAGCA
AGATTAAAATATAAGGTTCTTTAGCAGTTCTTAAGGCATATATGAATATTGCTGAAAAGGCTAAAATTGT
TAATGCTACTCCAGGAATACCACGCGTTGATAACGTATCTATGAGATCGTTATGCAAATGAGAATCTACA
AAGGGTAAAGCACCACTTAATCTAGGCTCTTTCTCTTCTAGTTCATGTATTTTTTCCGCACGTTTTTCCA
GTGATTGTCCTATTGGAGAATATGTTTTTAAACCAACTTCATACATTGCCAGACGGGCACCGACTGAAGT
TCTGGTGTTATCATGCGAATAATTAACTAAATCCCTATTTAAGTCATTCATTCTCGTAAGTAATGTATCT
TTAAAAGAAAATACACCTGCTAATAATGCAATCGCAATAAAACAATATAACTTATAATTAATTCGCTTAT
TAATATAACTATCCATTACGATTAATAGTAACGCAAAGAATGGAAACACTAATATGGCTGCACGCGTTTC
AGTTACTATAACAGCACATAAGCTAAGCATGAAGTTAATTGTATATAATGTTGTTTTATGTCTAAAGTCG
GATTTTAAAATGAGAATTGATGCCAGTAAAGAAGGGAAAAGGATGATGTAGCCTGCTGTTGTAGCGTGGG
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CTGCGTTGCCAAGGATGCGAACAGAATTAGTTTCCCAAATTTTATTTTGGTTCTCATCTCATCTTTAGCA
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TGGTATAAACCGAGCCTGAAACTTTATAATGTGAATACCAGATGATATTAACGATACCCAAAAGACATAA
TGAGATGAAAATTAACAAAGTAGGATTATTAGTTATTGTTTTTTTTGGTGTCGCTAGAAAATATAATGCA
GATATAATTCCAGCTGCATGAAGAAACTTTGTTGCAACATCATCCCACAAAGACAAAGTCATTAAAGACA
ACATACATAATATGAGAATTAAAGACTTGACACCAGAGTTAATTTCATTCCAACAAAACGACAT
```

Figure 4.1 Sequence of *waaL* gene: Sequence of gene retrieved from NCBI in FASTA format.

Size of *waaL* gene is 1.2kb

4.1.2 *In silico* gRNA/oligonucleotide design:

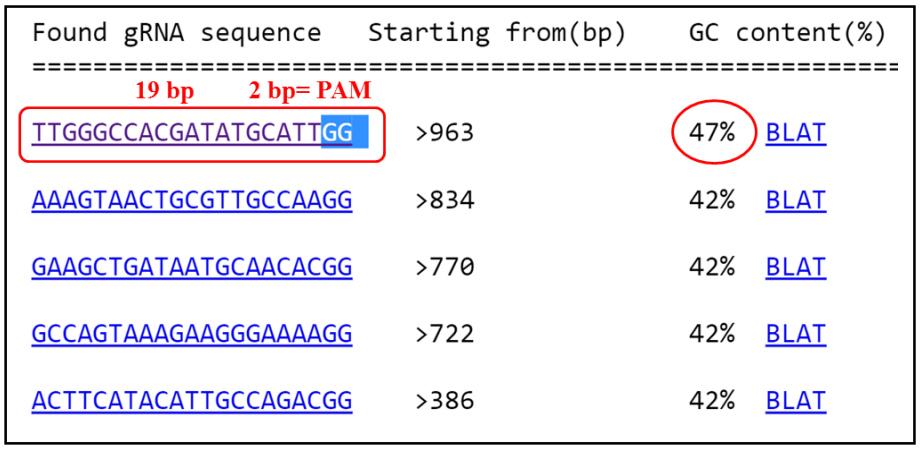


Figure 4.2 gRNA sequences found via Jack Lin's CRISPR/CAS gRNA finder: several gRNA sequences found with PAM sequence (NGG) with each gRNA located at the 5' end of each sequence.

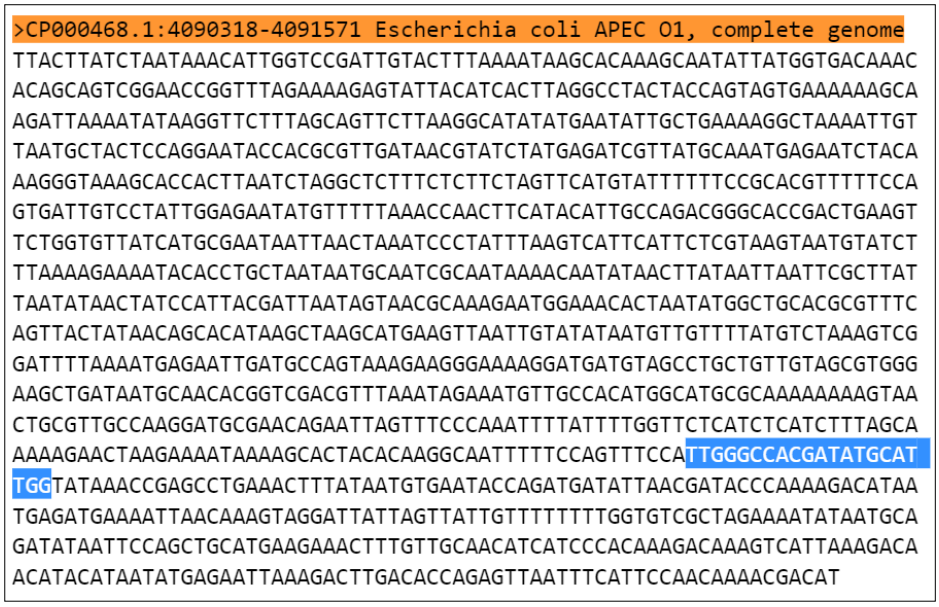


Figure 4.3 Location of gRNA in sequence of *waaL* gene: Highlighted region represents the location of gRNA sequence in *waaL* gene

For *in silico* gRNA designing, Jack Lin's CRISPR/CAS gRNA finder was used, and the most suitable gRNA sequence was selected because it has 40-70% GC content with minimum off target effects, and it lied in the functional part of the gene (Fig 4.2).

4.2. Wet Lab Results:

4.2.1. Confirmation of pCRISPR:

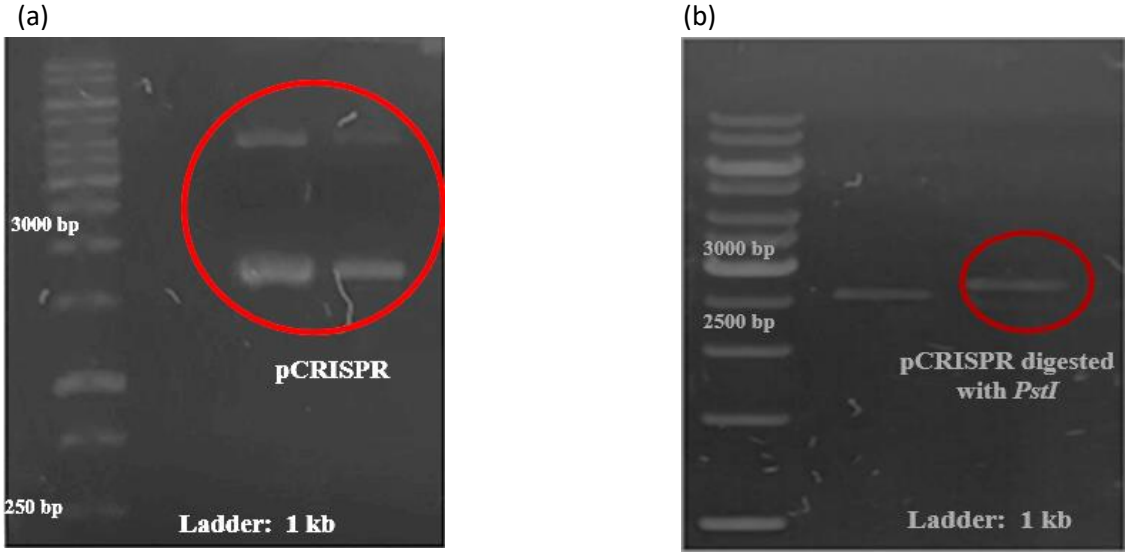


Figure 4.4 Confirmation of CRISPR plasmid: (a) 2.7 kb circular plasmid can be seen on the agarose gel while (b) shows the image of gel having bands of pCRISPR 2.7 kb digested with PstI enzyme.

Two plasmids were ordered from Addgene that were pCRISPR and pCAS9. The protocol for plasmid extraction mentioned in section 3.3.2 was followed in order to extract plasmids from the given strain of DH5α. For the confirmation of extraction, pCRISPR was digested with *PstI* restriction enzyme. For confirmation of plasmid extraction gel electrophoresis was performed, which showed band of 2.7kb (Fig 4.4).

4.2.2. Confirmation of Cas9 Plasmid:

Isolation of Cas9 plasmid was done by the miniprep method mentioned in section 3.3.2. After plasmid extraction pCAS9 was digested with *BsaI* restriction enzyme in order to verify the plasmid via gel electrophoresis, which showed band of 9.3kb in (Fig 4.5).

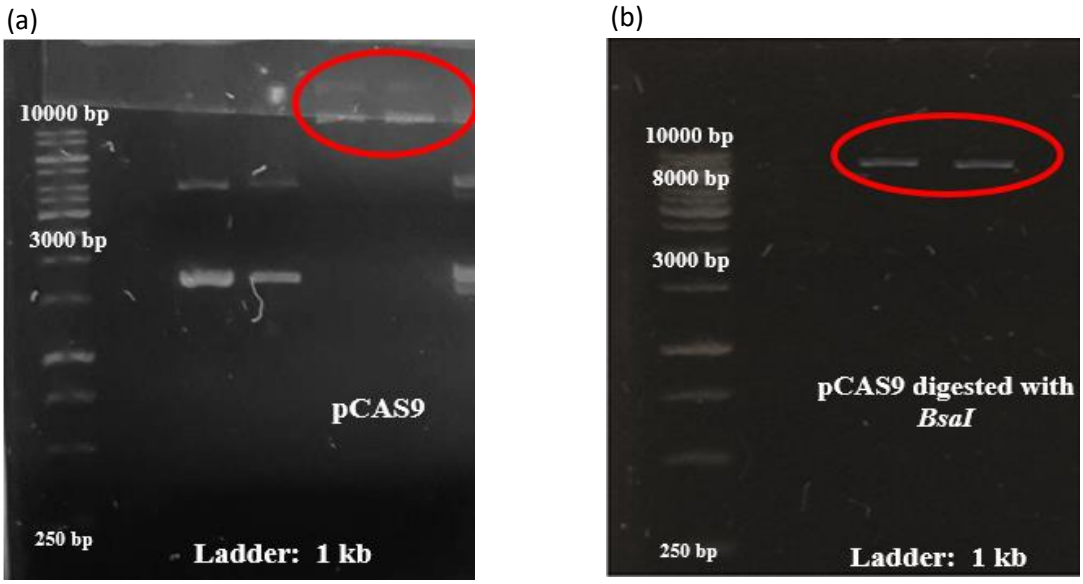


Figure 4.5 Verification of Cas9 plasmid: (a) shows bands of undigested pCAS9 and (b) shows the picture of gel showing bands of pCAS9 digested with BsaI enzyme observed under UV Illuminator.

4.2.3. gRNA dimerization and confirmation:

In order to clone the gRNA sequence into the expression vector i.e. pCRISPR, double stranded oligonucleotide was required. For that purpose, forward primer (our designed gRNA) and reverse primer (complimentary sequence of gRNA) (Table 3.28) were ordered from Macrogen, Korea. The two primers were annealed and phosphorylated according to the method described in section 3.3.4, which were further inserted in *BsaI* digested pCRISPR. Successful annealing of forward and reverse primer (oligos) to from a primer-dimer was confirmed by gel electrophoresis (Fig 4.6).

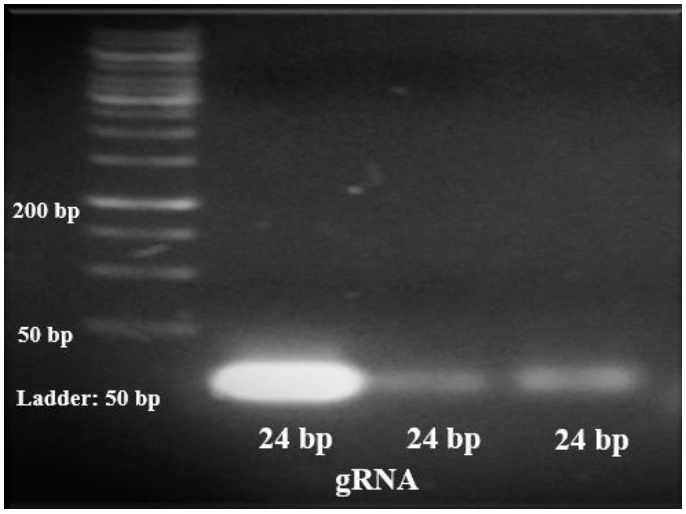


Figure 4.6 gRNA formation in the form of primer dimer: The image of the gel shows formation of gRNA in the form of primer dimer showing size of 24 bp

4.2.4. Transformation of Ligated Vector

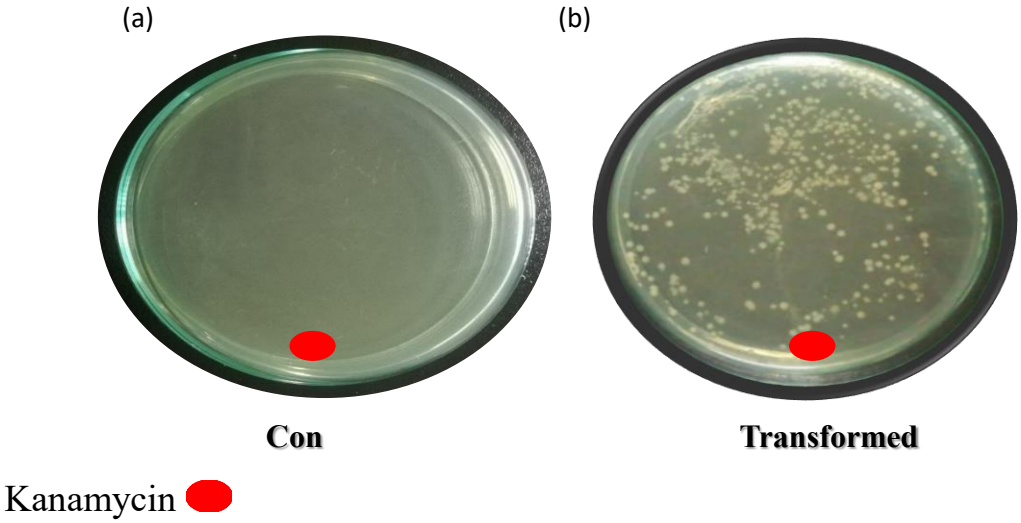


Figure 4.7 Results of transformation of ligated vector: The plate on right (b) shows the transformed colonies on the kanamycin antibiotic plate since pCRISPR has kanamycin as its selection marker whereas in (a) no colonies were seen on the control plate having kan

In order to ligate annealed oligonucleotides i.e. gRNA into pCRISPR, first pCRISPR was digested with restriction enzyme *BsaI* as explained in 3.3.5 followed by gel purification of digested vector

mentioned in segment 3.3.6. Ligation of gel purified pCRISPR and gRNA was done as mentioned in section 3.3.8 later followed by competent cell preparation of DH5α and heat shock transformation. Results showed presence of colonies on the kanamycin containing plate (Fig 4.7) which were further confirmed by colony PCR.

4.2.5. Confirmation of pCRISPR+ gRNA by Colony PCR:

After the observation of colonies on antibiotic plates which indicated the successful single transformation. These preliminary results were confirmed by colony PCR mentioned in section 3.3.11. Verification of amplification by colony PCR was done by gel electrophoresis. Genotypic confirmation by colony PCR confirmed successful ligation of gRNA into pCRISPR (Fig 4.8).

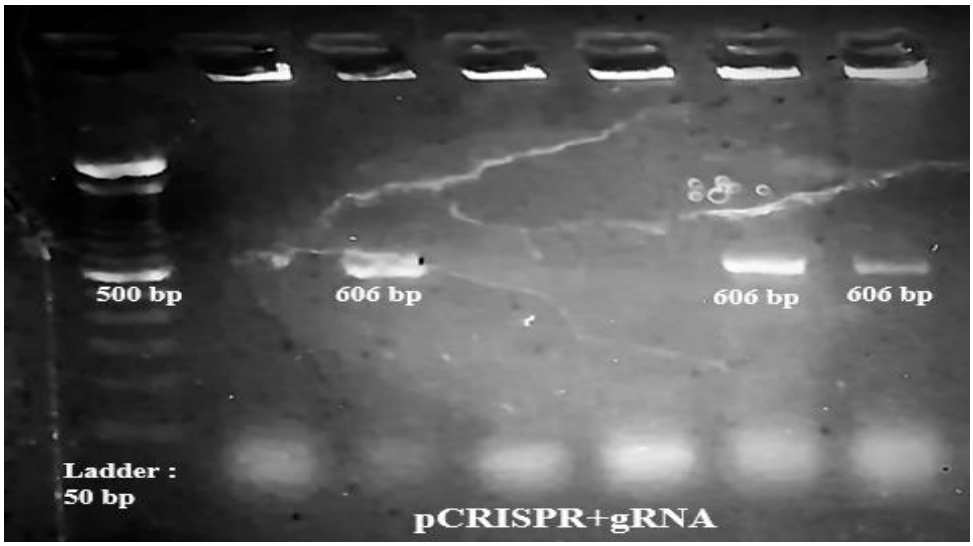


Figure 4.8 Confirmation via colony PCR: image of gel showing vector and insert showing the amplicon size of 606 bp.

4.2.6. Co-transformation of pCRISPR+gRNA and pCas9 into APEC O1:

After confirmation of pCRISPR + gRNA, next step was transformation of the ligated vector with pCas9 into APEC O1 and for that co-transformation was needed. As it was bit difficult with conventional chemical competent cells and heat shock method, so that's why approach of

electroporation was deployed. First electro-competent cells were prepared as mentioned in section 3.3.12 followed by electroporation method explained in 3.3.13. The next day we got colonies on the antibiotic containing plates which confirmed co-transformation (Fig 4.9).

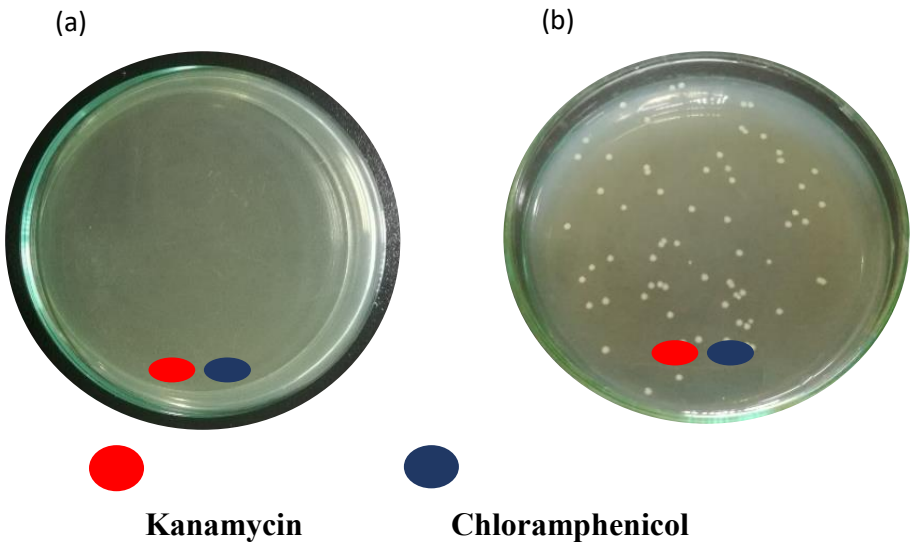


Figure 4.9 Results of co-transformation: The plate on right (b) shows the co-transformed colonies has its selection marker and pCAS9 has chloramphenicol. On the left (a) no colonies were seen on the control plate having both antibiotics as selection markers.

4.2.7. Confirmation of co-transformation via colony PCR:

Initially, co-transformation was confirmed by assessment of colonies on the antibiotic plates having kanamycin and chloramphenicol as selection markers. However, subsequent confirmation was done by colony PCR whose details are mentioned in 3.3.14. One set of Primer was designed inside the gRNA and the other was designed in the downstream region of the gene. Initially the idea was the confirmation of knockouts from false clones but the results of gel (Fig 4.10) were vague despite of having significant differences in pathogenicity results and based on these results best performing strains were sent for sequencing along with WT-O1.

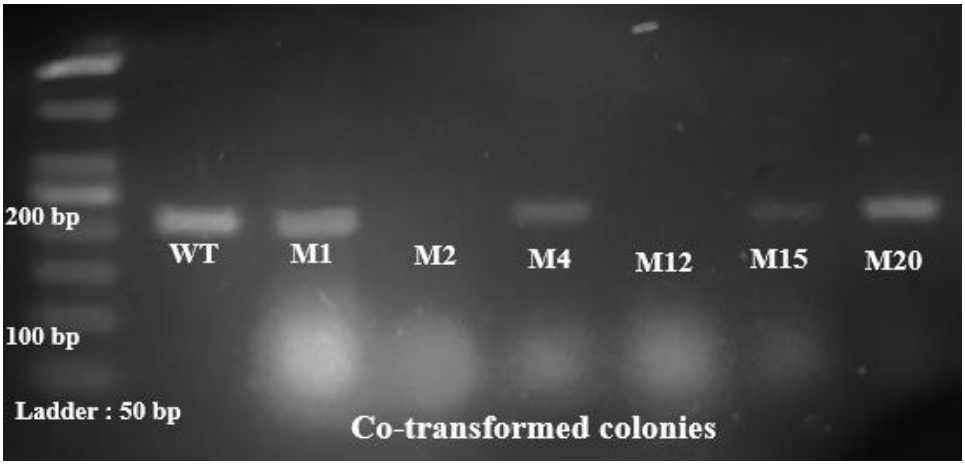


Figure 4.10 Confirmation of co-transformation via colony PCR: Gel electrophoresis image showing co-transformed colonies obtained via colony PCR

4.2.8. Results of sequencing for confirmation of gene knockout:

Figure 4.11 Results of sequencing of M12 compared with WT-O1: sequence of M12-O1 when compared to WT-O1 showed removal of 13 nucleotide indicating the complete frameshift.

In order to confirm the gene knockout, PCR product of *waaL* gene of WT-O1, M1-O1and M12-O1 were sent for sequencing. The results of sequencing for M12-O1 (Fig 4.11) indicated the removal of 13 nucleotides thus indicating complete frameshift whereas sequence of M1-O1

compared with WT-O1 (Fig 4.10) showed mutations in upstream and downstream of the region where gRNA was designed.

Figure 4.12 Sequencing result of M1-O1 in comparison with WT-O1: sequence of M1-O1 in comparison with WT-O1 showed mutations in upstream and downstream of gRNA.

4.2.9. Growth Curve

In order to see whether there was any difference in the normal growth rate of WT-O1 compared to M1-O1 and M12-O1, growth rate of the bacterial strains was measured. Growth rate at different time intervals ranging from 0 hrs. to 96 hrs. was recorded by checking OD at 600nm and growth Curve was plotted afterwards which showed there was no difference in the growth rate of M1-O1 and M12-O1 as compared to WT-O1 (Fig 4.13).

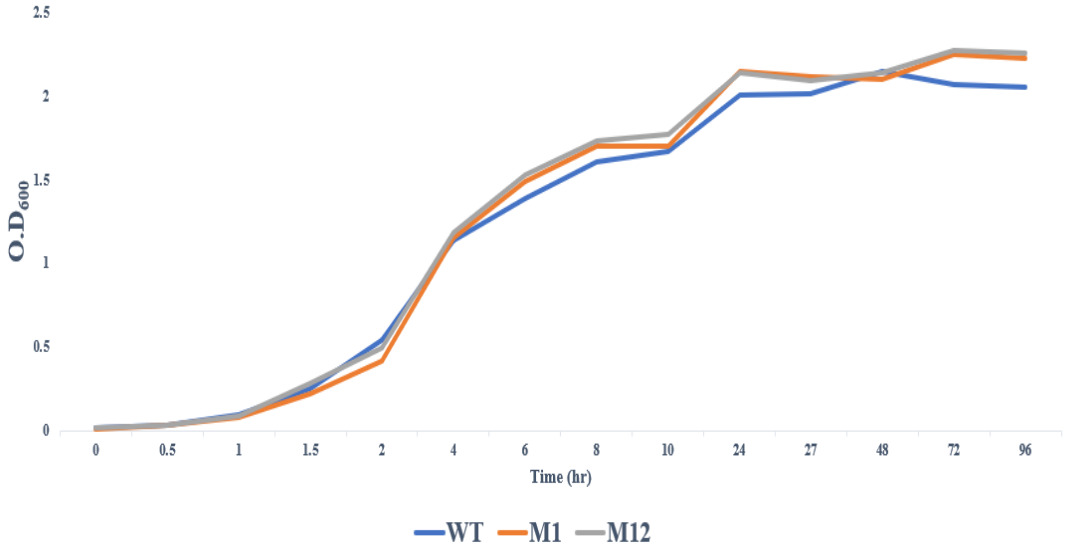


Figure 4.13 Growth Curve of bacterial strains: the growth curve showing the growth rate intervals from 0 hrs. to 96 hrs. X-axis shows time interval in hours while Y-axis shows O.D at 600nm, showing no difference in growth rate of two mutants M1-O1 and M12-O1 when

4.3. Analysis of the survival rate under different physiological stress conditions

Unlike the growth curve under normal conditions which showed no effect in case of WT-O1 when compared to M1-O1 and M12-O1 (Fig 4.12), the same strains when exposed to different physiological stress conditions showed different results.

4.3.1. Osmolarity Endurance:

In order to observe survival rates of WT-O1, M1-O1 and M12-O1 in high osmolarity, bacteria were subjected to high salt concentration of 4.8M NaCl and incubated at 37ºC for 1 hour as mentioned in section 3.5.1. Results showed that there was decrease in growth (CFU/ml) for M1-O1 and M12-O1 when compared to WT-O1 (Fig 4.14).

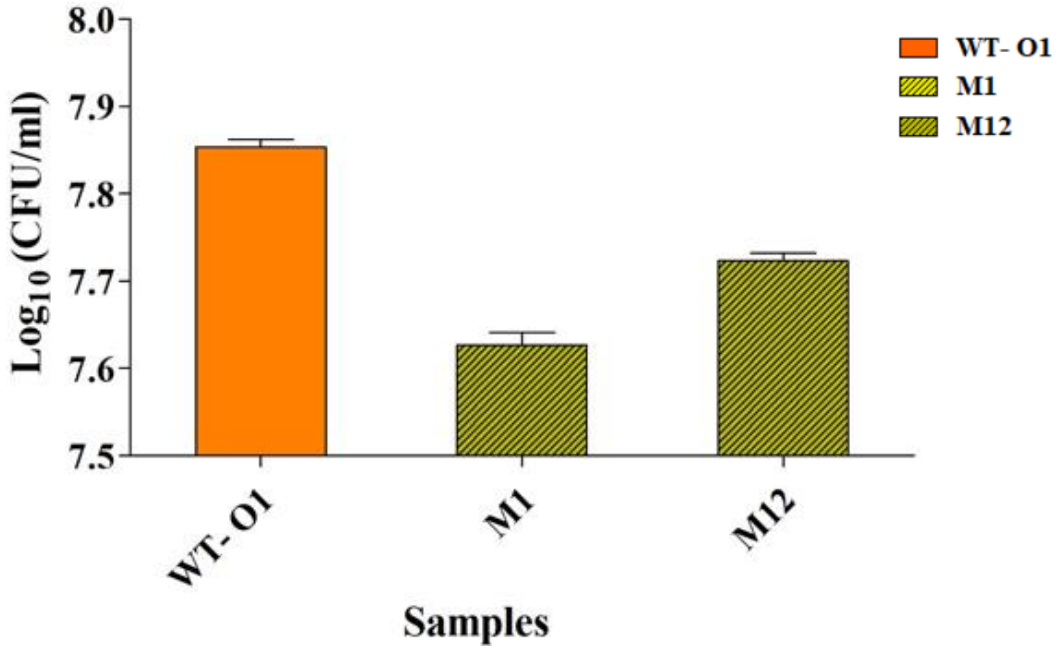


Figure 4.14 The bar graph showing osmolarity endurance rate: X-axis shows bacterial samples while Y-axis shows CFU/ml in Log10. Graph showing decrease in growth from 7.85 for WT-O1 to 7.71 for M12-O1and for M1-O1, a value of 7.63 was recorded.

4.3.2. Heat Shock

For checking heat shock endurance, the bacterial cells were given heat shock at 54ºC for 3 min and they were plated as mentioned in section 3.5.2. Heat treated samples showed decreased growth for M12 and M1 as compared to WT-O1 (Fig 4.15).

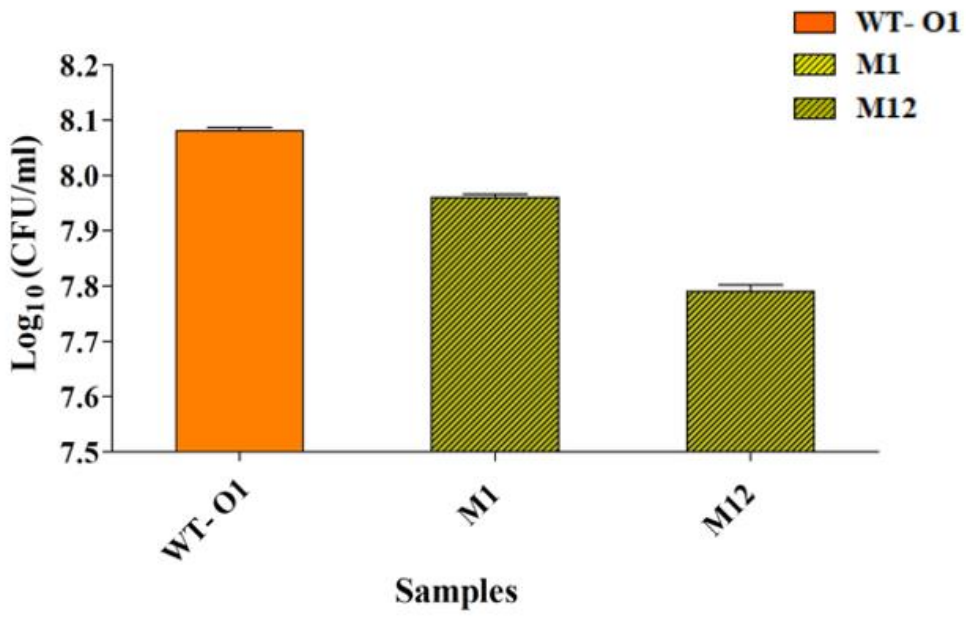


Figure 4.15 Bacterial survival rate under different physiological stress conditions: Bar graph showing the effect on endurance rate of bacterial samples when exposed to heat shock showing samples on X-axis while growth rate in CFU/ml in log10 showing growth rate of WT-O1 which was 8.05 and M-12 showing a decrease to 7.8.

4.3.3. Acid Endurance:

For acid endurance, the pH of media was adjusted at 3.00 and incubated at 37ºC for 20 min followed by method of serial dilution and plating as explained in section 3.5.1. Results showed the decrease in the growth rates ranging from 6.82 of WT-O1 to 6.55 for M12-O1 (Fig 4.16).

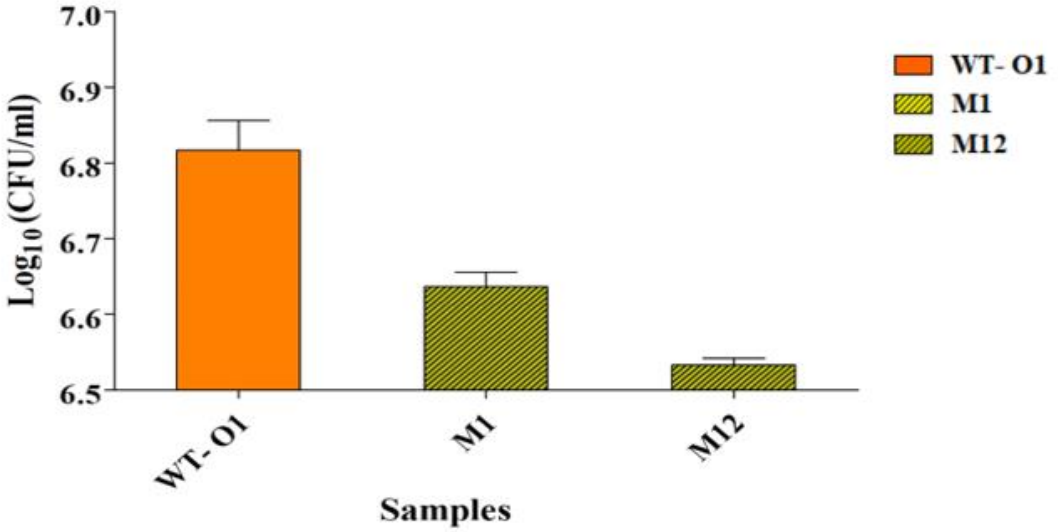


Figure 4.16 The bar graph showing bacterial endurance rate of bacterial samples when subjected to acid endurance: The graph shows decrease in growth rate of M1-O1 and M12-O1 as compared to WT-O1

4.3.4. Alkali Endurance:

In order to evaluate the bacterial alkali endurance, the pH was adjusted at 10:00 as mentioned in 3.5.4 and the results showed decrease in growth rate of M1-O1 and M12-O1 as compared to WT-O1 (Fig 4.17) thus indicating that deletion of *waaL* gene decreased resistance of APEC O1 to different physiological stress conditions.

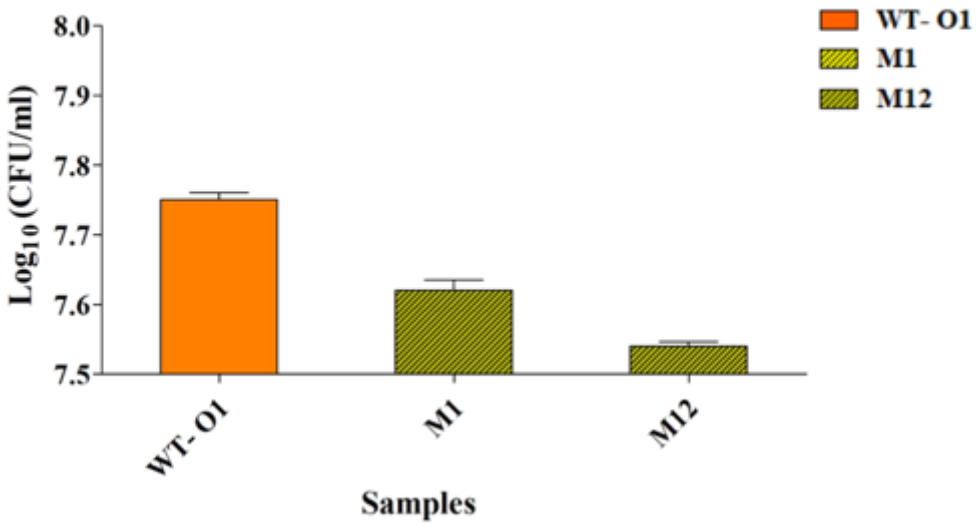


Figure 4.17 Bacterial endurance rate under different physiological stress conditions: Bar graph showing bacterial growth rate under alkali conditions: The graph shows decrease in growth rate of M1-O1 and M12-O1 as compared to WT-O1

4.3.5. Biofilm quantification via Microtiter Assay for Wild type and Mutants:

E. coli are generally not good biofilm formers but as biofilm enhances pathogenicity of bacteria therefore, we wanted to know if deletion of *waaL* gene has affected the biofilm forming ability or not. Biofilm forming ability was estimated by 96 well microtiter plate by using the biofilm assay. After 48hrs of incubation, planktonic and loosely attached cells were removed, and tightly bound cells were first fixed and stained with CV solution. The results showed that there was a slight decrease in biofilm of the mutants M1-O1 and M12-O1 compared to WT-O1. This is an indication for the role of *waaL* gene in biofilm formation. (Fig 4.18).

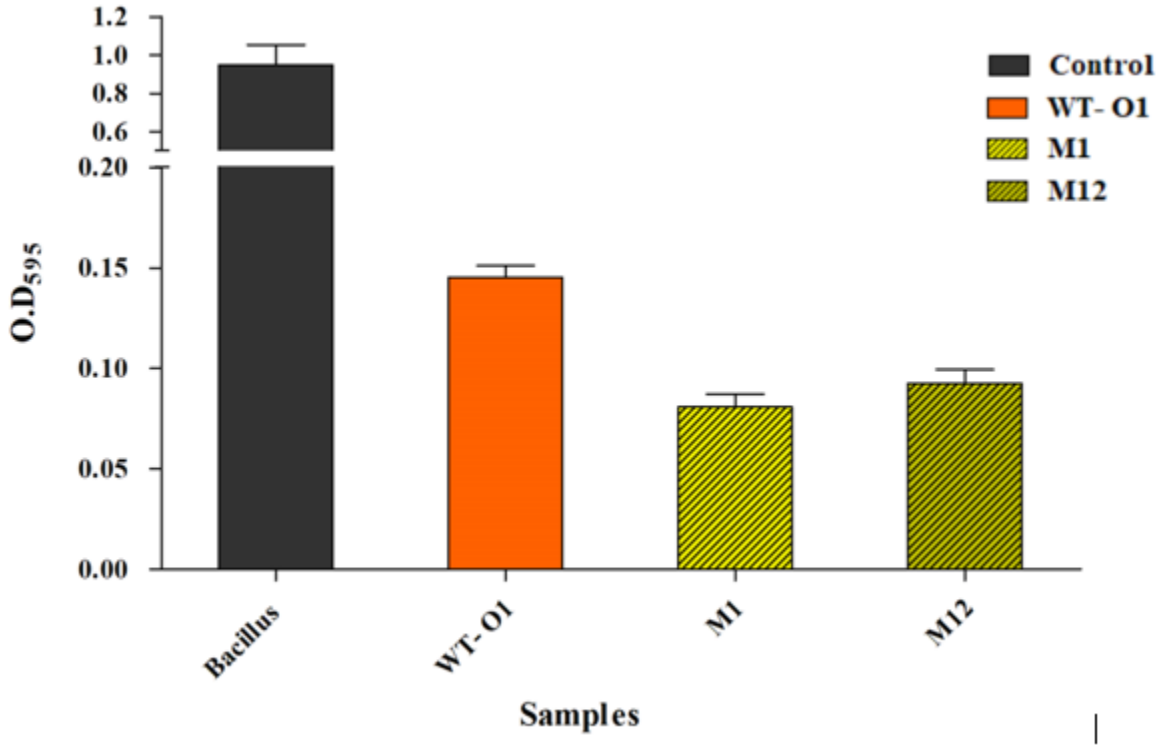


Figure 4.18 Biofilm forming ability of bacterial samples in bar graph: Bacillus sp. was taken as a positive control. WT-O1, M1-O1 and M12-O1 are shown on X-axis while O.D 595nm shown on Y-axis.

4.3.6. Confirmation of the zoonotic potential of the mutant vs WT strains:

Non-pathogenic *E. coli* are not capable of growing in highly acidic and nitrogenous environment like urine. Only UTI causing *E. coli* strains has this ability to grow in Urine. So, in order to check the survival of APEC in human urine, growth in human urine test was performed. The test gives an estimation of bacterial growth in urine (bacteriuria), indicating zoonotic potential of the respective bacteria. O.D of bacterial samples and positive control (UPEC) and negative control (DH5α) were compared with the O.D of sterile urine after 8 hrs. of incubation. The results showed that there was decreased growth of mutant strains M1-O1 and M12-O1 as compared to WT-O1 (Fig 4.19) thus indicating possible role of *waaL* gene in zoonosis.

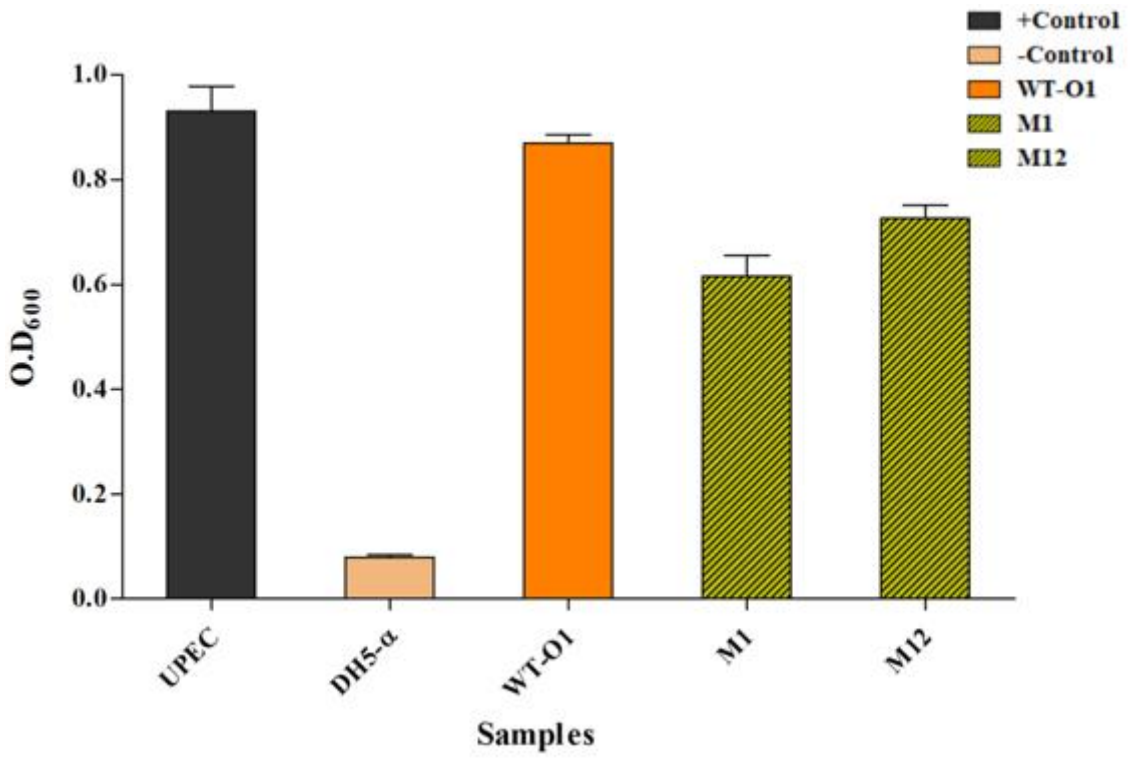


Figure 4.19 The bar graph showing O.D of wild type and mutants after 8-hour incubation in human urine: UPEC was taken as positive control and DH5α as negative control.

4.3.7. Motility Assays of Wild type and Mutants of APEC O1:

Many bacteria can move in response to availability of nutrients and it has been found that motile bacteria are more pathogenic as compared to non-motile. Hence motility assays were performed in order to check the effect of deletion of *waaL* gene on motility.

4.3.8. Swimming Motility:

Swimming motility is the movement of single bacterium in liquid medium powered by flagella. Swimming motility of WT-O1, M1-O1 and M12-O1 were qualitatively analysed as described in 3.8.1. Results showed decrease in zone size of M12-O1 which was 5mm in comparison with WT-O1 which showed zone size of 12mm on swimming agar plates (Fig 4.20)

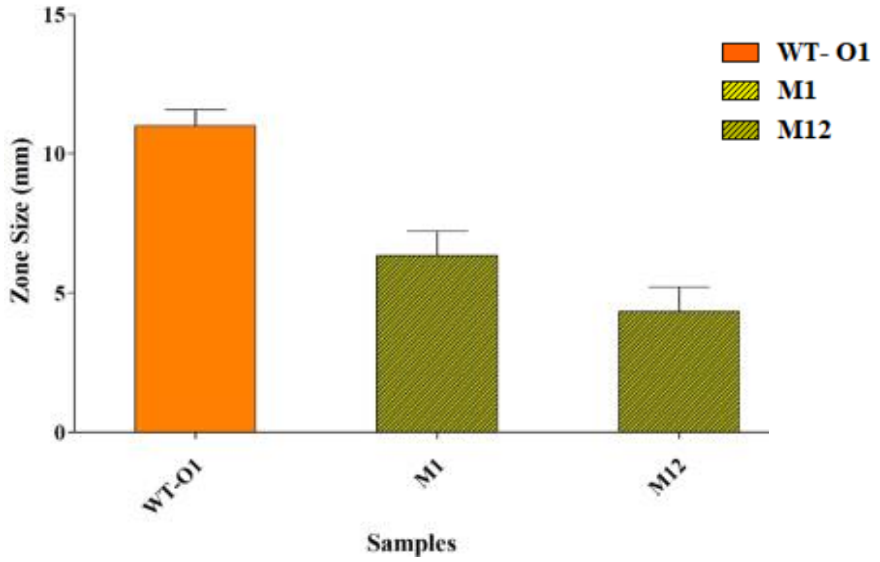


Figure 4.20 Swimming motility assay for wild type and mutants: Bar graph showing samples on X-axis and Y-axis shows the zone size in mm thus presenting the zone size of WT-O1 was 12mm and on the other hand the zone size decreased to 5mm for M12-O1

4.3.9. Swarming Motility:

Swarming motility is movement of group of bacteria in semi solid medium. Swarming motility of WT-O1, M1-O1 and M12-O1 were qualitatively analysed according to the method described in 3.8.2. The results showed decrease in zone size of M12-O1 which was 5mm as compared to zone size of WT-O1 which was recorded as 13mm (Fig 4.19).

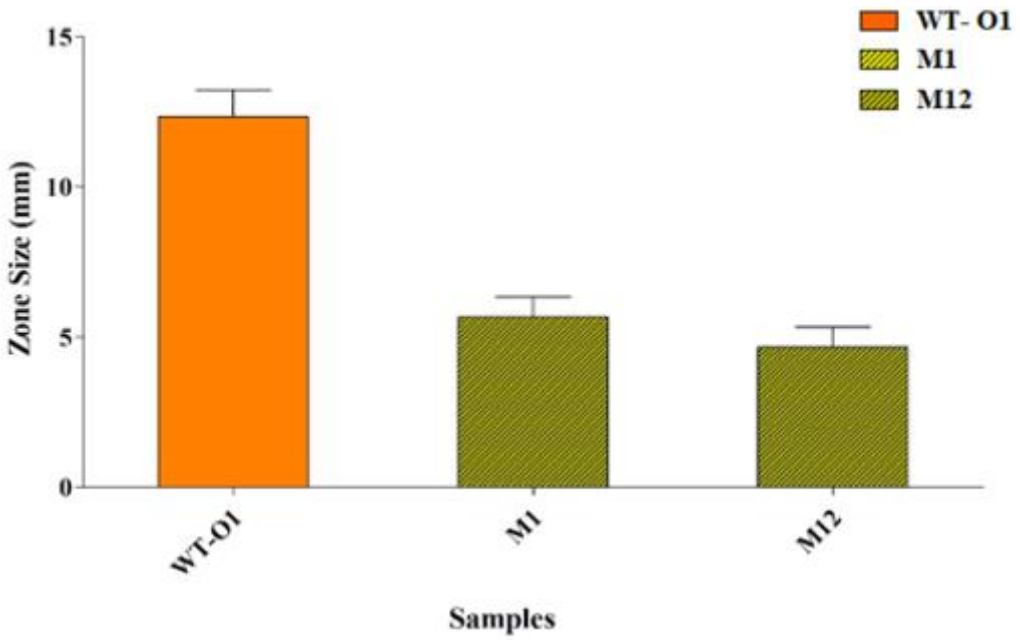


Figure 4.21 Swarming motility for wild type and mutants: Bar graph showing Samples on X-axis and Y-axis shows the zone size in mm thus presenting the zone size of WT-O1 was 13mm and on the other hand the zone size decreased to 5mm for M12-O1.

4.3.10. Twitching Motility:

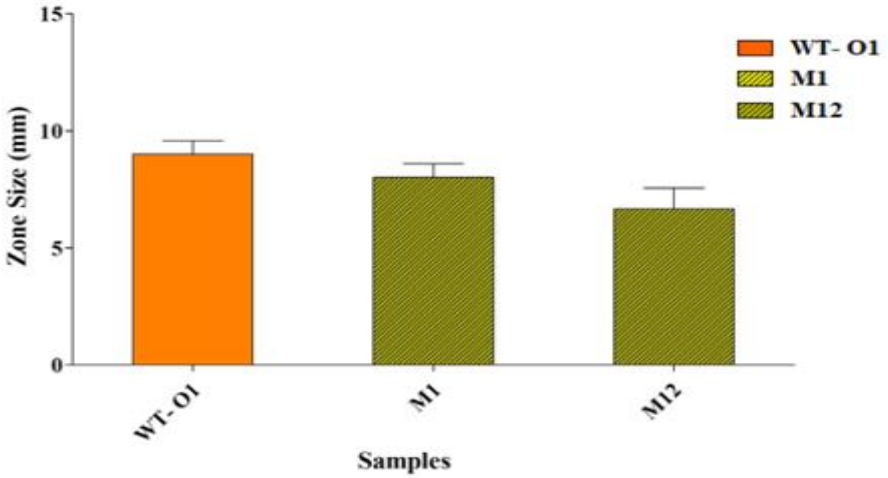
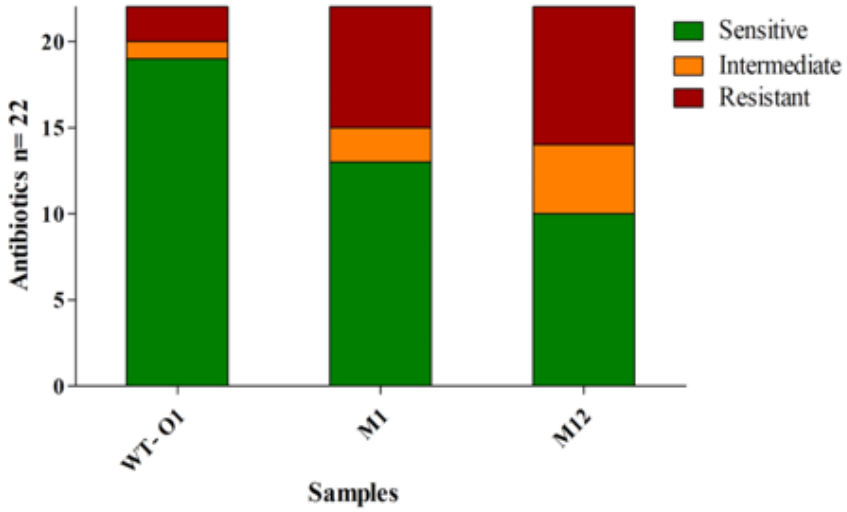


Figure 4.22 Twitching motility for wild type and mutants: Bar graph showing Samples on X-axis and Y-axis shows the zone size in mm thus showing that there was no significant difference in the zone sizes of WT-O1, M1-O1 and M12-O1.

Twitching motility is movement of group of bacteria powered by retractive movement of pili on solid medium. Twitching motility of WT-O1, M1-O1 and M12-O1 were qualitatively analysed by (Qiao, Li, Xu, & Park, 2012) as described in 3.8.3. However, results showed no significant differences in the zone sizes of WT-O1 when compared with the results of M1-O1 and M12-O1 (Fig 4.23)

4.3.11. Antibiotic Susceptibility testing of Wild type and Mutants:



Sensitive	Intermediate	Resistant
19	1	2
13	2	7
10	3	9

Figure 4.23 Antibiogram: WT-O1 showed resistance against 2 antibiotics whereas M1-O1 showed resistance against 7 antibiotics and the resistance pattern kept on increasing for M12-O1 which showed resistance against 9 antibiotics.

APEC WT-O1, M1-O1and M12-O1 were subjected to antibiotic susceptibility testing to various commonly used antibiotics against *E. coli*. WT-O1 showed resistance against Meropenem (10), Erythromycin (15) and intermediate resistance against Levofloxacin (5). Whereas the resistance pattern kept on increasing for M1-O1 showed resistance against Tetracycline(30), Meropenem(10), Streptomycin(10), Ciprofloxacin(5),Levofloxacin(5), SXT(25) and Erythromycin(15) and intermediate resistance against Norfloxacin(10), and M12-O1 showed

resistance against Amoxicillin(10), Ampicillin(10), Doxycycline(30), Tetracycline(30), Meropenem(10), Gentamicin(10),Streptomycin(10), SXT(25) and Erythromycin(15) and showed intermediate resistance against Tobramycin(10), Ciprofloxacin (5) and Levofloxacin(5) (Fig 4.24).

Class	Antibiotics	WT- O1	M1	M12
Penicillin	AML (10)	S	S	R
	AMP (10)	S	S	R
Penicillin-β-lactamase inhibitor	AMC (30)	S	S	S
	TZP (110)	S	S	S
Tetracycline	DO (30)	S	I	R
	TE (30)	S	R	R
Carbapenem	ETP (10)	S	S	S
	MEM (10)	R	R	R
	IPM (10)	S	S	S
Aminoglycoside	CN (10)	S	S	R
	TOB (10)	S	S	I
	S (10)	S	R	R
	AK (30)	S	S	S
Fluoroquinolone	CIP (5)	S	R	I
	NOR (10)	S	I	S
	LEV (5)	I	R	I
	SXT (25)	S	R	R
Nitrofuran	F (300)	S	S	S
Macrolide	E (15)	R	R	R
Cephalosporin	CRO (30)	S	S	S
	CTX (30)	S	S	S
	CAZ (30)	S	S	S

Figure 4.24 Antibiotic resistance pattern for wild type and mutants: the image showing the details of 22 antibiotics used for WT-O1, M1-O1 and M12-O1

5. Discussion

Avian colibacillosis is pondered amongst significant bacterial diseases of poultry and serves as one of the major causes of mortality and morbidity in poultry industry with high economic losses throughout the globe. Various genes which have been involved in playing roles in the pathogenicity of APEC include genes related to regulation, adhesion, invasion, bacterial metabolism and LPS biosynthesis (Janßen, Schwarz, Preikschat, Voss, Philipp, & Wieler, 2001). Outer membrane in Gram negative bacteria is made up of LPS which acts as an endotoxin. LPS is comprised of three definite parts: lipid A, core oligosaccharide and O-antigen. There are number of genes that are involved in synthesis of O-antigen and the lipid A-core ligase, *waaL* gene is amongst one of them (Wang & Quinn, 2010). Several reports revealed the virulence property of *waaL* gene by signature-tagged mutagenesis (Li, Laturnus, Ewers, & Wieler, 2005). Despite all this information, the role of *waaL* gene in the pathogenicity of APEC still needed more investigation.

The focus of this study was the investigation of *waaL* gene in the pathogenicity of APEC. To understand this, *waaL* gene was targeted using CRISPR Cas9 genome editing tool which resulted in the gene knockout and two mutant strains M1-O1 and M12-O1 were proceeded for further analysis. Strain M1-O1 showed mutations in the upstream and downstream of the region where gRNA was designed while 13 nucleotides were deleted in strain M12-O1 at the exact location where the gRNA was designed.

The mutants M1-O1 and M12-O1 showed almost same growth curve as compared to WT-O1 under normal conditions which was in accordance with the already reported study in which *waaL* gene mutant strains showed similar growth pattern compared to the WT strains (Y. Han, Han, Wang,

Meng, Zhang, Ding, & Yu, 2014). Whereas on the other hand endurance rates to different physiological stress conditions varied. Mutant M1-O1 and M12-O1 were found to be more sensitive to heat shock, alkali and acid endurance compared to WT-O1 and the possible reason behind this decrease in endurance rates of mutants as compared to WT-O1 and that is because LPS plays an important role in the integrity of APEC and has been already reported that interference of LPS structure has been found responsible for decreased bacterial resistance to various environmental stress (Ernst et al., 1999). These results agreed to already reported results in which *waaL* gene mutant strains showed decreased endurance rates as compared to wild type to different physiological stress conditions (Y. Han, Han, Wang, Meng, Zhang, Ding, & Yu, 2014). Its noteworthy to mention that certain genes have been reportedly found on large plasmids in APEC that are known to occur on UPEC plasmids (Sorsa et al., 2003) and pathogenicity islands (PAIs) as well (Dobrindt et al., 2001; Oelschlaeger et al., 2002). In another study UPEC and APEC showed considerable overlap regarding their phylogenetic groups and serogroups and on the basis of these findings, tendency of APEC to spread to human beings and the ability of APEC as a source of virulence genes to UPEC should be considered for their contribution to uropathogenic (Rodriguez-Siek et al., 2005). Zoonotic risk of APEC was investigated by performing growth of the mutants and WT strains in human urine and results suggested that WT-O1 have the potential to survive and grow in urinary bladder and might have zoonotic potential as well. This was in accordance with the already reported studies which reported that APEC can cause disease in mammalian models that imitate human UTI (Manges & Johnson, 2012). The. zoonotic risk of APEC was originally linked to a verity that few human and avian EXPEC share virulence genes and similar phylogenetic background (Manges & Johnson, 2012). Interestingly, mutants M1-O1

and M12-O1 showed decreased growth as compared to WT-O1 and this indicate *waaL* gene involvement in pathogenicity (Y. Han, Han, Wang, Meng, Zhang, Ding, & Yu, 2014).

It has been already reported that biofilm formation is a must prerequisite for pathogenicity (MacKenzie et al., 2017). Biofilm quantification of WT-O1, M1-O1 and M12-O1 resulted in decrease biofilm forming ability of M1-O1 and M12-O1 when compared with WT-O1 which was in contradiction to the already reported study where deletion of *waaL* gene resulted in an increased biofilm formation (Han *et al*., 2014). One possible reason behind the decrease in biofilm forming ability of mutants M1-O1 and M12-O1 lies in the fact that *waaL* gene is involved in the LPS synthesis which play a pivotal role in providing structural integrity to the outer membrane of gram-negative bacteria (Nikaido and Vaara, 1985) and deletion of *waaL* gene might contributed towards the instability of the structural stability of LPS thus indicating the involvement of *waaL* gene in pathogenesis of APEC (Y. Han, Han, Wang, Meng, Zhang, Ding, & Yu, 2014).

Additionally, decreased swimming and swarming motility resulted in the decreased virulence of the mutant strains M1-O1 and M12-O1 which is consistent with the previous studies that motility plays an important role in bacterial pathogenesis and infection (Josenhans & Suerbaum, 2002).

It's distinctly intriguing to consider the results of antibiotic susceptibility testing (AST). When WT-O1, M1-O1, and M12-O1 were subjected to commonly used antibiotics, the results of M1-O1 and M12-O1 were quite interesting, instead of becoming sensitive they became resistant, which was in agreement with already reported study which demonstrated in impulsive resistance to fluroquinolone (norfloxacin or ciprofloxacin) could be an effect of a punctiform mutation of the two genes *gyrA* and *gyrB* that codes two protein subunits of an enzyme, DNA gyrase that lead to changes in the structure of gyrase that lead to absence of binding ability for fluroquinolone (Willmott & Maxwell, 1993). Similarly, antibiotic resistance against fluroquinolones,

tetracyclines, aminoglycosides, and penicillin was enhanced in mutant strains indicating mutation in *waaL* gene and suggesting that that *waaL* gene knockout might be helping another cell survival pathway.

This study provided valuable insight on an under investigated reason of pathogenicity of APEC infection which accounts for huge issues inside poultry sector. This study revealed the function of *waaL* gene which is involved in LPS synthesis in APEC which agrees with the already reported study which accounted *waaL* gene responsible for the LPS synthesis and pointing its role in pathogenicity of APEC (Y. Han, Han, Wang, Meng, Zhang, Ding, & Yu, 2014). Decrease in biofilm forming ability might be because deletion of *waaL* gene might be causing disturbance in the structural stability of LPS. Deletion of the target gene lead to decreased motility, decreased bacterial endurance rates under different stress conditions thus indicating reduced virulence for the mutant strain ΔwaaL (Y. Han, Han, Wang, Meng, Zhang, Ding, & Yu, 2014). Reduced growth of mutant strains M1-O1 and M12-O1 in human urine as compared to WT-O1 indicate zoonotic potential of *waaL* gene. Increased antibiotic resistance hypothesize that *waaL* gene knockout might be helping some other cell survival pathways thus suggesting *waaL* gene knockout might be contributing towards the loss of virulency of APEC which is in harmony with another study in which deletion of the *ibeA* gene resulted in decreased ability in spreading *in vitro* and less virulency *in vivo* (Wang & Quinn, 2010).

6. Conclusion

The study conducted was to investigate and confirm the possible involvement of an LPS coding gene, *waaL* in the APEC strain. After the successful knockout of *waaL* gene by CRISPR-Cas9 two mutants M1-O1 and M12-O12 were obtained. *In vitro* pathogenicity results revealed involvement of *waaL* gene in the pathogenicity of APEC. Normal growth was observed to be unaffected in mutant strains under normal condition, but bacterial endurance rate reduced significantly under different physiological stress conditions. A decrease in motility and biofilm forming ability was also reported which indicates the possible connection of *waaL* gene with bacterial pathogenicity. Reduced growth of mutant strains, M1-O1 and M12-O1, in human urine compared to WT-O1 suggests that *waaL* gene might be a possible factor in zoonosis potential of APEC. Moreover, increased antibiotic resistance of the mutant strains indicated that *waaL* gene knockout might be helping some other cell survival pathways.

7. Future Prospects

In order to keep a track on the various stages of genome editing workflow, Next generation sequencing (NGS) may be utilized at various stages i.e. from confirmation of CRISPR knockouts towards the analysis of off-target effects and assessment of the functional impacts of gene edits. Evaluation of the *waaL* gene product can be done via RT-PCR and western blot to confirm turnover of mutant gene. For better investigation of *waaL* gene involvement in pathogenicity of APEC *in vivo* testing of the mutants M1-O1 and M12-O1 along with wild-type WT-O1 in chicken models is needed. For the further elucidation regarding the role of *waaL* gene in zoonosis, comparative analysis of the APEC and UPEC *waaL* genes should be carried out. Since colibacillosis is one of the major health concerns to poultry industry as well as humans so in order to develop therapeutic strategy multiple genes can be targeted at once. CRISPR Cas9 genome editing technique can be utilized for selective targeting of the bacterial populace based on the gRNA sequence used, thus preventing loss of useful micro biota in return.

8. References

Ahmad, I., Anjum, M. S., & Hanif, M. (2012). Prevalence of poultry diseases at high altitudes of district Poonch Azad Jammu & Kashmir. *Pakistan Journal of Science, 64*(4).

Alexander, C., & Rietschel, E. T. (2001). Bacterial lipopolysaccharides and innate immunity. *J. Endotoxin Res, 7*, 167-202.

Ceasar, S. A., Rajan, V., Prykhozhij, S. V., Berman, J. N., & Ignacimuthu, S. (2016). Insert, remove or replace: a highly advanced genome editing system using CRISPR/Cas9. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1863*(9), 2333-2344.

Chang, A. Y., Chau, V. W., Landas, J. A., & Pang, Y. (2017). Preparation of calcium competent Escherichia coli and heat-shock transformation. *JEMI Methods, 1*, 22-25.

Clermont, O., Christenson, J. K., Denamur, E., & Gordon, D. M. (2013). The C lermont E scherichia coli phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environmental microbiology reports, 5*(1), 58-65.

Croxen, M. A., & Finlay, B. B. (2010). Molecular mechanisms of Escherichia coli pathogenicity. *Nature Reviews Microbiology, 8*(1), 26-38.

Dobrindt, U., Blum-Oehler, G., Hartsch, T., Gottschalk, G., Ron, E. Z., Fünfstück, R., & Hacker, J. (2001). S-Fimbria-Encoding Determinant sfa I Is Located on Pathogenicity Island III536 of UropathogenicEscherichia coli Strain 536. *Infection and immunity, 69*(7), 4248-4256.

Ernst, R. K., Guina, T., & Miller, S. I. (1999). How intracellular bacteria survive: surface modifications that promote resistance to host innate immune responses. *The Journal of infectious diseases,, 179*(2), 326-330.

Genetics Home Reference. (2018). What are genome editing and CRISPR-Cas9? *Help Me Understand Genetics Genomic Research.* Retrieved from https://ghr.nlm.nih.gov/primer/genomicresearch/genomeediting

Han, X., Bai, H., Liu, L., Dong, H., Liu, R., Song, J., . . . Yu, S. (2013). The luxS gene functions in the pathogenesis of avian pathogenic Escherichia coli. *Microbial pathogenesis, 55*, 21-27.

Han, Y., Han, X., Wang, S., Meng, Q., Zhang, Y., Ding, C., & Yu, S. (2014). The *waaL* gene is involved in lipopolysaccharide synthesis and plays a role on the bacterial pathogenesis of avian pathogenic Escherichia coli. *Veterinary microbiology, 172*(3-4), 486-491.

Heinrichs, D. E., Monteiro, M. A., Perry, M. B., & Whitfield, C. (1998). The Assembly System for the Lipopolysaccharide R2 Core-type ofEscherichia coli Is a Hybrid of Those Found inEscherichia coli K-12 and Salmonella enterica STRUCTURE AND FUNCTION OF THE R2 WaaK AND WaaL HOMOLOGS. *Journal of Biological Chemistry, 273*(15), 8849-8859.

Hille, F., & Charpentier, E. (2016). CRISPR-Cas: biology, mechanisms and relevance. *Philosophical transactions of the royal society B: biological sciences, 371*(1707).

Ipe, D. S., & Ulett, G. C. (2016). Evaluation of the in vitro growth of urinary tract infection-causing gram-negative and gram-positive bacteria in a proposed synthetic human urine (SHU) medium. . *Journal of microbiological methods, 127*, 164-171.

Janßen, T., Schwarz, C., Preikschat, P., Voss, M., Philipp, H. C., & Wieler, L. H. (2001). Virulence-associated genes in avian pathogenic Escherichia coli (APEC) isolated from internal organs of poultry having died from colibacillosis. *International Journal of Medical Microbiology, 291*(5), 371-378.

Johnson, T. R., & Case, C. L. (1992). Laboratory Experiments in Microbiology. *The Benjamin/Cummings Publishing Company, Inc*.

Josenhans, C., & Suerbaum, S. (2002). The role of motility as a virulence factor in bacteria. *International Journal of Medical Microbiology, 291*(8), 605-614.

Kearns, D. B. (2010). A field guide to bacterial swarming motility. *Nature Reviews Microbiology, 8*(9), 634-644.

Kucheria, R., Dasgupta, P., Sacks, S., Khan, M., & Sheerin, N. (2005). Urinary tract infections: new insights into a common problem. *Postgraduate medical journal, 81*(952).

Li, G., Laturnus, C., Ewers, C., & Wieler, L. H. (2005). Identification of genes required for avian Escherichia coli septicemia by signature-tagged mutagenesis. *Infection and immunity, 73*(5), 2818-2827.

Linton, A. H., HOWE, K., Bennett, P. M., Richmond, M. H., & Whiteside, E. J. (1977). The colonization of the human gut by antibiotic resistant Escherichia coli from chickens. *Journal of Applied Bacteriology, 43*(3), 465-469.

MacKenzie, K. D., Palmer, M. B., Köster, W. L., & White, A. P. (2017). Examining the link between biofilm formation and the ability of pathogenic Salmonella strains to colonize multiple host species. *Frontiers in veterinary science, 4*, 138.

Manges, A. R., & Johnson, J. R. (2012). Food-borne origins of Escherichia coli causing extraintestinal infections. *Clinical infectious diseases, 55*(5), 712-719.

Marraffini, L. A., & Sontheimer, E. J. (2010). CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nature Reviews Genetics, 11*(3).

Mattingly, A. E., Weaver, A. A., Dimkovikj, A., & Shrout, J. D. (2018). Assessing Travel Conditions: Environmental and Host Influences on Bacterial Surface Motility. *Journal of Bacteriology*(11), 14-31.

Montminy, S. W., Khan, N., McGrath, S., Walkowicz, M. J., Sharp, F., Conlon, J. E., . . . Akira, S. (2006). Virulence factors of Yersinia pestis are overcome by a strong lipopolysaccharide response. *Nature immunology, 7*(10), 1066-1073.

Oelschlaeger, T. A., Dobrindt, U., & Hacker, J. (2002). Pathogenicity islands of uropathogenic E. coli and the evolution of virulence. *International journal of antimicrobial agents,, 19* (6), 517-521.

Pitout, J. (2012). Extraintestinal pathogenic Escherichia coli: a combination of virulence with antibiotic resistance. *Frontiers in microbiology, 3*, 9.

Qiao, G., Li, H., Xu, D. H., & Park, S. I. (2012). Modified a colony forming unit microbial adherence to hydrocarbons assay and evaluated cell surface hydrophobicity and biofilm production of Vibrio scophthalmi. *ARS USDA Submissions, 3*(1).

Raimondi, S., Righini, L., Candeliere, F., Musmeci, E., Bonvicini, F., Gentilomi, G., . . . Rossi, M. (2019). Antibiotic resistance, virulence factors, phenotyping, and genotyping of E. coli Isolated from the Feces of Healthy Subjects. *Microorganisms, 7*(8), 251.

Rehman, H., Fawad, N., Abbas, G., Naheed, G., Siddique, B., Afzal, F., . . . Haq, I. (2013). Surveillance of poultry diseases in Punjab Province, Pakistan; special reference to Newcastle disease. *Res J Vet Pract,, 1*, 1-4.

Rodriguez-Siek, K. E., Giddings, C. W., Doetkott, C., Johnson, T. J., Fakhr, M. K., & Nolan, L. K. (2005). Comparison of Escherichia coli isolates implicated in human urinary tract infection and avian colibacillosis. *Microbiology, 151*(6), 2097-2110.

Sander, J. D., & Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature biotechnology, 32*(4), 347.

Sorsa, L. J., Dufke, S., Heesemann, J., & Schubert, S. (2003). Characterization of an iroBCDEN gene cluster on a transmissible plasmid of uropathogenic Escherichia coli: evidence for horizontal transfer of a chromosomal virulence factor. *Infection and immunity, 71*(6), 3285-3293.

Stepanović, S., Vuković, D., Dakić, I., Savić, B., & Švabić-Vlahović, M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of microbiological methods, 40*(2), 175-179.

Tenaillon, O., Skurnik, D., Picard, B., & Denamur, E. (2010). The population genetics of commensal Escherichia coli. *Nature Reviews Microbiology, 8*(3), 207-217.

Van Elsas, J. D., Semenov, A. V., Costa, R., & Trevors, J. T. (2011). Survival of Escherichia coli in the environment: fundamental and public health aspects. *The ISME journal, 5*(2), 173-183.

Wang, X., & Quinn, P. J. (2010). Lipopolysaccharide: biosynthetic pathway and structure modification. *Progress in lipid research, 49*(2), 97-107.

Willmott, C. J., & Maxwell, A. (1993). A single point mutation in the DNA gyrase A protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex. *Antimicrobial agents and chemotherapy, 37*(1), 126-127.