An M13 Phagemid Mediated Therapy to Silence Virulence Factors of Avian Pathogenic *E. coli* (APEC)

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2019

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This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science

in

Industrial Biotechnology

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2019

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National University of Sciences & Technology

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Sayed Abdullah

Dedicated to

My beloved parents

especially my Mother

Acknowledgements

First, I owe it all to Almighty God for granting me the wisdom, health and strength to undertake this research task and enabling me to its completion.

I would like to express my sincere gratitude to my supervisor Dr. Fazal Adnan for the continuous support of my master study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my master study.

Besides my advisor, I would like to thank the rest of my GC member: Dr. Muhammad Faraz Bhatti, Dr. Saadia Andleeb, and Dr. Abid Ali Khan for their insightful comments and encouragement, but also for the hard questions, which incented me to widen my research from various perspectives.

I am thankful to my class-mates and specially my research fellows; Mehk, Maryam, Maheen, Nigham Sarfaraz, Sufayn, Waqar, Agha and all those I didn't mention here for providing a friendly environment, sharing hard days with no results and no bands on the gel, being supportive to each other's problems and the good days we enjoyed last three years.

I am also thankful to my seniors specially Amna Jalil and my juniors; Asim Zahoor and Qurat ul Ain for being supportive and helped out at lab work.

Last but not the least; I would like to thank my family, especially my mother for her support and encouragements.

Thanks for all your encouragement!

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List of Abbreviations

MDR: Multi drug resistance

APEC: Avian Pathogenic *E. coli*

ExPEC: Extra-intestinal Pathogenic *E. coli*

NMEC: Neonatal Meningitis *E. coli*

RT PCR: Real Time Polymerase Chain Reaction

sRNA: Small/ Short RNA

ncRNA: Non Coding RNA

UTI: Urinary Tract Infection

RISC: RNA Induced Silencing Complex

NDM: New Delhi Beta-Lactamase

HGT: Horizontal Gene Transfer

RBS: Ribosomal Binding Site

UTRs, Untranslated Regions

UPEC: Uropathogenic *E. coli*

LB: Luria Bertani

BIMs: Bacteriophage Insensitive Mutants

Abstract

Avian Pathogenic *E. coli* (APEC) causes various poultry infections, which leads to high rate of mortality and morbidity in poultry flocks. The current treatment strategies against APEC infections involve antibiotics and vaccines but they are becoming ineffective due to resistance and the emergence of new variants of APEC. Novel strategies have evolved to treat bacterial infection and to combat multidrug resistant bacteria. These novel strategies involve phage therapy, phagemid mediated therapy, probiotics, nanoparticles, quorum quenching and antimicrobial peptides. Phage therapy is one of the promising strategies to treat bacterial infections but a bit of modifications in phage genome via genetic engineering can broaden the host range, reverse antibiotic resistance and using phages to deliver sRNAs, antibiotics and antibodies into the target host. The phagemid-mediated therapy comprises of phagemid, which include sRNA expression cassette that contains pre-designed GUIDE sequences for the target gene. The M13 helper phages deliver the phagemids to the target host, achieved by the presence of f1 origin of replication. This study involved the designing of GUIDE sequence for pre-designed sRNA expression cassette, computationally analysis of sRNA binding energy, secondary structures and off target predication. Results showed that the designed sRNA has binding energy of -29.60kcal/mol with zero off targets. The real time RT-PCR showed that the *csgA* gene was repressed about 43% in phagemid treated APEC-O1 as compared to wild type APEC-O1. Similarly, the biofilm was reduced up to 34% in *csgA* silenced APEC-O1 compare to wild type APEC-O1. A little higher spreading was observed in *csgA* silenced APEC-O1 as compared to wild type APEC-O1, which showed that curli fimbria restrained the flagellar motility and tends to colonization. The study needs to be further investigated for the infection efficiency of M13 helper phages *in vivo*, using chick model. Furthermore, it is also important to search for other targets (genes) to eliminate or reduce the pathogenicity at higher level.

Chapter 1:

1 Introduction

Antimicrobial resistance is a global issue from decades, however the geographic zone affected by drug resistance, the breadth of resistance in single organisms as well as number of resistance organism are extraordinary and growing. Infections and infectious agents that were once supposed to be controlled by antibiotics are recurring in new groups resistant to these treatments. As the resistance of antibiotics within organism increases, render the effect of therapy more hazardous and expensive and sometimes ineffective. Many developing countries have yield to multi drug resistance (MDR) infections as a result of individual resistance to all available drugs [1, 2]. The bacterial infections have become a threat again after when first patient was treated with antimicrobial drugs specially antibiotics because of antibiotic resistance [3]. The MDR, known global examples include community and hospital MDR strains of *Escherichia coli (E. coli)*, *Staphylococcus aureus (S. aureus)*, *Mycobacterium tuberculosis*, *Enterobacter cloacae*, *Klebsiella pneumonia (K. pneumonia)*, *Acinetobacter baumanii*, *Enterococcus faecium* and *Pseudomonas aeruginosa* [2, 4, 5]. *E. coli* is the main cause of urinary tract infection (UTI) and resistance has shown to six member of drug families and fluoroquinolones. It should be noted that in some parts of southeast China and Asia [2] and some developed countries and the United States, the resistance is approaching about 10% are also alarming, because the tendency endangered the value of drug family [6, 7]. The quick rise of bacterial resistance worldwide has jeopardized the efficiency of antimicrobials, which has rehabilitated drugs and saved millions of peoples [8-10].

The mismanagement and overdoing of these medications and in addition, the decrease in new drug advancement by the pharmaceutical industries due to challenging regulatory requirements and less economic inducements has led to antibiotics resistance crisis [11, 12]. A high morbidity and mortality have been noted, as the antimicrobial resistance has increased globally. The conventional antibiotics become ineffective due to patterns of multidrug resistance in gram-negative and gram-positive and led to challenging to treat and even fatal infection. Early reporting of microbes and their pattern of antimicrobial sensitivity in patients with septicemia and other sever infection is absent in several health-care setting with

abundant and most unnecessarily use of broad-spectrum antibiotics is the main cause of antibiotic ineffectiveness [13].

Resistances against antibiotics is due to antibiotic resistances genes, which ineffective antibiotics to protect themselves from killing. The well-known antibiotic resistance gene produced, called NDM-1(new dehli-metallo-beta-lactamase) enzyme, present in some of gram-negative bacteria of Enterobacteriaceae family (*E. coli*, and *K. pneumoniae*) which led to produce resistance against almost all beta-lactams antibiotics group, including carbapenems [11]. Most of studies also observed that, it is not specifically the genes which are responsible for antibiotic resistance, are significantly met in clinical pathogens, though somewhat all commensal, environmental and most frequently pathogenic bacteria, bacteriophages, transposable elements, gather to create pool of antibiotics resistance genes (resistome), where pathogenic bacteria obtain resistance via horizontal gene transfer. It is important to understand the level of pool of antibiotic resistance genes and its moment to pathogenic antibiotics. where it takes place, to control the spreading of these resistance genes among microflora [14].

In addition to antibiotic resistance genes, a different strategy of pathogenic bacteria is biofilms formation, making the antibiotics ineffective. Biofilms is one of great global challenge due to the antibiotic resistance inherited and conserved within pathogenic bacteria existence. Combating against biofilms frequently requires high dose of antibiotics for a prolong time, these methods contributing to infection persistence rather than effectively work against it. In addition to ineffectiveness of therapeutic treatments, biofilms can grow in medical device, so it can be the source of infection. These worldwide challenges imposed by biofilms has prepared researchers globally to propose or advance alternative antimicrobial therapies to control biofilms [15].

Recent epidemiological updated data availability on antibiotic resistance of bacterial pathogens will be significant and not just to decide treatments strategies, but also in hospital, to develop an efficient antibiotics Stewardship program [13]. To combat against bacterial Infection and accompanied disease, many challenges such as the recent scarcity of effective drugs, ineffectiveness of conventional drugs, deficiency of effective prevention measures and insufficient antimicrobials within clinical pipeline, need an advancement in alternative remedies possibilities and new antibiotic therapies [16]. In alternatives, non-coding RNAs

(ncRNA) can be efficient strategy to minimize the virulence, antibiotic resistance or infection causing genes to bypass the resistance.

The ncRNA have gained a huge interest as universal regulator almost in all division of life. The microRNAs and short interfering RNAs of the eukaryotes have been making great development during last few years. Currently, with the experimental and computational approach and development, several hundred of sRNAs have been found in bacteria especially in *E. coli* [17, 18]. sRNAs mediated knock out is extensively used to regulate specific target gene expression at translational level. This adoptable class of techniques eases to make use of several short RNAs that can be easily produced from any sequence and design to match a part of any gene targeted for silencing. Due to easy introduction of sRNA construct into many cell types using various vectors or directly (modified sRNAs) and genes can be knock downed *in vivo* without laborious genetic engineering and modification. RNA interference is known to be the most common RNA silencing technology, utilize RNA induce silencing complex to facilitate identification and degradation of the target mRNAs. However, This technique is limited to RNA Induced Silencing complex (RISC) expression host, founds in eukaryotes mainly [19].

There is enormous class of Trans-encoded sRNAs, which regulate their target mRNAs that is mediate by Hfq protein. Hfq is a rich RNA chaperon protein that binds the mRNAs in a number of case studies [20-22]. Base pairing of sRNA with the target mRNAs is assisted by Hfq protein that enhances the sRNAs stability *in vivo*, by protecting them from nucleases degradation. Hfq protein is reported to interact with several proteins such as RNase E, poly (A) polymerase and polynucleotides phosphorylase, which are involved in mRNA degradation and form fibers *in vitro*, while the physiological importance of fibers is still unknown [23-27]. In contrast to gram-negative bacteria, it was unnecessary for sRNA-mediated regulation that it would requires Hfq protein in gram-positive bacteria for long time. However, some recent studies provided strong evidence for Hfq-dependent antisense regulation in *Listeria monocytogenes*, a gram-positive bacterium [28, 29].

Phagemid therapy utilized synthetic sRNAs or ncRNAs, which may consider an efficient therapy in future. In this therapy, the synthetic sRNAs were expressed to silence the virulence genes and genes involve in infections and diseases. Different RNA knock-downing methods

have shown a down regulation of a specific genes about 50-90% [30, 31], while the phagemid therapy has shown 80% down regulation of the target gene [20]. Our study focus on the targeting of virulence gene (*csgA*) of the APEC O1 by synthetic sRNAs silencing and the observation of the reduction of virulence in target host and efficiency of synthetic ncRNAs silencing. The strategy has already used against CAT gene and which restore the sensitivity of chloramphenicol antibiotic in *E. coli* MG1655 K12 strain [20].

Aim of the study is to find the phagemid efficiency to silence virulence gene (*csgA*), to minimize the pathogenicity, using M13 phagemid delivery system an applicable and feasible therapy. Objectives of the studies were, *in silico* designing of GUIDE sequence for the pre-designed sRNA, production of M13 packaged phagemid and phage transduction into target APEC-O1 host and lastly, expression analysis of silenced gene. The expression analysis of silenced gene was analyzed by using Real time RT-PCR, biofilm assay, motility assay, hemagglutination assay and hemolysis assay.

Chapter 2:

2 Literature Review

2.1 Antibiotic's Origin

The finding of antibiotics has updated the treatment and control of infectious diseases, into clinical practices, which were previously hard to treat [32]. The first discovered antibiotic was Sulfonamides in 1937, first ever efficient antimicrobial, which was used as a therapeutic drug at that time. The growth of sulfonamides resistance was first reported at late 1930s and persisted for over seven decades [33], until Penicillinase was discovered since 1940s as the first antibiotic resistance encounter to penicillin, known as the world's most effective medicine, founded by Fleming in 1928. An adequate amount of classed antibiotics has been developed and industrially produced after that and use of antibiotic concurrently increased with the resistance of antibiotics [34].

2.2 Antibiotic Resistance Development

The discovery of the antibiotics (magic bullet) was not only to treat bacteria, however as soon as the new antibiotics were discovered, its resistance also emerged. Primarily, it was supposed that the different frequency of mutations in bacteria made them super-bugs to many antimicrobial agents [35]. Unfortunately, at first, nobody anticipated that microbes would create resistance to antibiotics, but due to the changing environment and bacterial nature, they were acknowledged of using a wide variety of mechanisms. Surprisingly, the ability of interchanging genes between bacteria by horizontal gene transfer (HGT) was also discovered. It was later found that resistance was developed even before the first antibiotic, penicillin, was discovered. The β-lactam drugs were first identified in *E. coli*, before the distribution of penicillin for use as therapeutic medicine [35]. Beside from β-lactam, the aminoglycoside antibiotics was one of the effective antibiotics that encountered resistance to many infections [36]. Over time, it became clearer that the increase and miss-use of antibiotics contributed to the rise of antibiotic resistance [37]. Bacteria have developed resistance through a number of mechanisms which include; change of permeability due to the bacterial cell wall mutation in the efflux pumps, modification of antibiotic by enzymes, inactivation of the drugs through degradation, acquisition of resistance genes and antibiotic target modifications over-

production of enzyme involved in targeting. The time line of antibiotic resistance from 1943-2011 is depicted in figure 1 [11].

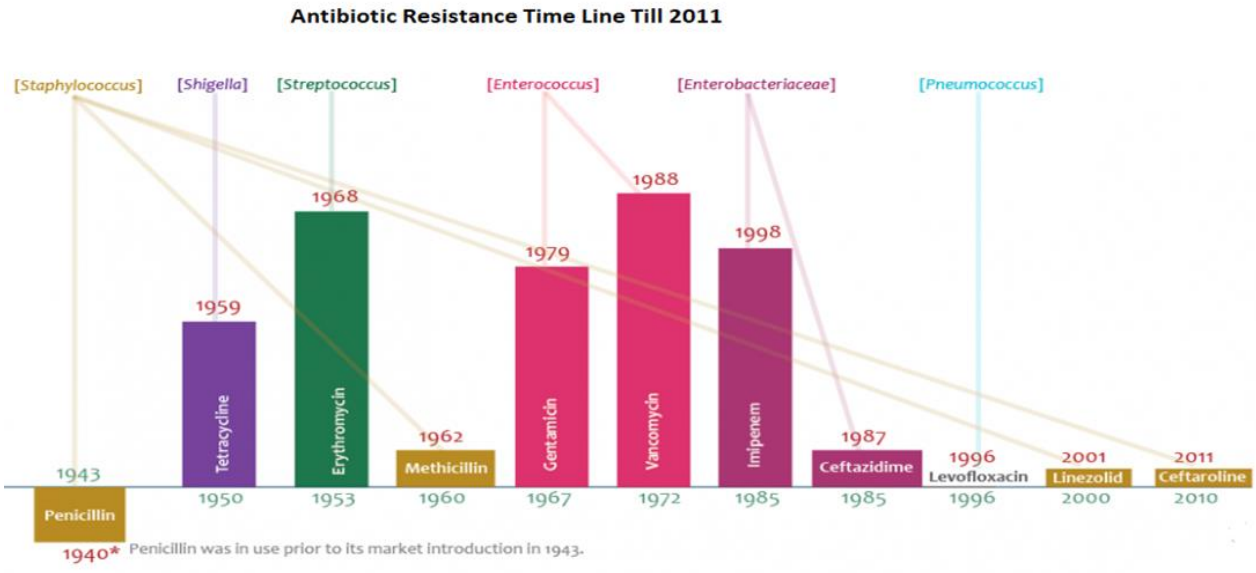

Figure 1: Time line of antibiotic resistance of bacteria starts from 1940 until 2011

2.3 Introduction to APEC

E. coli is a gram negative, non-acid fast staining, uniform, rod shape bacillus that can grows both anaerobically as well as aerobically, present in different shapes and size. *E. coli* is non-spore forming and many of strains found to be motile with a peritrichous flagella. *E. coli* counts in a normal microflora of poultry intestine and some of *E. coli* strains are the known cause serious disease such as Colibacillosis known as systemic deadly disease [38-40]. Pathogenic strains of *E. coli* to poultry belongs to several serogroups, predominantly to O1, O2 and O78, and up to some extent O55 and O15 [41, 42].

2.4 Virulence Factors of APEC

Several genes with a potential virulence were recognized in APEC and their properties associated with virulence and comprise of respiratory tract adhesion, resistance against immunological defenses, cytotoxic effects involvement and proliferation under iron restricted condition [42]. A significant loss of poultry industry was found to be the reason by *E. coli* infection in several parts of the world. The involvement of virulence factor and mechanism of pathogenesis are yet not fully explained [43]. There are many virulence genes which include

type 1 fimbriae causes adhesion, iron scavenging, temperature sensitive haemagglutinin (*Tsh* gene) and the protection (*Iss* gene) are known virulence factors that allow many intestinal commensal *E. coli* make them APEC and leads to extra-intestinal site infection [38].

Most of these virulence genes have role in enabling of APEC strain to last in host fluids, adherence as well as resistance to host immune defense. These are often found to be present in Colicin-V (ColV) type or other large size plasmids found in *E. coli* [44-46]. P-fimbriae causes adherence to internal organs, it also protects *E. coli* from neutrophil's antibacterial action [47]. Similarly, the Type I fimbriae is largely dispersed in strains of APEC and found that it could easily bind to the cells of tracheae of avian and deliberate their abilities [48, 49].

2.4.1 Adherence:

E. coli isolates of mammals were found positive for the type I fimbriae, it is claimed that type I fimbriae is involved in the first step of colonization in the bird's air sacs and lungs. Deeply the type I fimbriae was reported to bind to mannose structure, however glycosylated protein found in avian cells, certain by this adhesion has not recognized since [43]. The *csg* genes encoded curli fimbriae, are thin and curly adjuncts, found on the Salmonella spp. And *E. coli* cell surface [50], these appendices are responsible for the bacterial connection to extracellular matrix and proteins [51] and for external environment adjustment and bacterial survival [52]. It is reported that curli fimbriae expression associated genes consist of two operons: *csg*ABC and *csg*DEF. The *csgA* encoded the major subunit of curli fimbriae known as the curlina, the monomer's structural subunit. The *csgB* gene is responsible for the expression of protein required for stability of curlina complex [40].

2.4.2 Temperature-sensitive Hemagglutinin:

Tsh is a virulence gene, that encodes a protein called atuo-transporter, having maximum homology to subclass of the family of *IgA* protease. It was found that *Tsh* has role in the adherence mechanism of the respiratory tract of avian [53]. *Tsh* encoded protein is a bifunctional protein, with proteolytic and adhesive activities [54]. *Tsh* encoded protein responsible for hemagglutination activity with chicken erythrocytes showed highest expression at 26ºC [55].

2.4.3 Iron Acquisition System:

APEC strains grow and survive inside a host with a low iron availability, due to present of Iron Acquisition System [56]. Bacterial iron uptake mechanism involves production of siderophores that act as ion chelants inside the host [57]. There are two main type of siderophores; hidroxamate and fenolates. Hidroxamate siderophore is known as Aerobactin that is encoded by *iuc*ABDC operon present in the plasmid [58-60]. Similar system the yersiniabactin system was present in many isolates of APEC strains in a higher frequency, comprise of *fyuA* and *irp-2* genes [43, 61-63]. The *iucA* and *fepC* are involve in other types of iron acquisition system were also found in many isolates of avian *E. coli* strain [62, 64]. It has been investigated that aerobactin iron-uptake system has great role in the virulence of APEC. Prevalence studies have shown that more that 80% of APEC isolates were positive for aerobactin iron-uptake genes [65, 66]. It was observed that knockout of the aerobactin encoded genes operon showed reduction in the virulence of APEC strains [67].

2.4.4 Colicin:

APEC strains are reported positive for Col-V plasmid [68]. These plasmids contain colicin a bacteriocin that kills other bacteria. In addition to colicin these plasmid also comprise of other pathogenicity related genes [62, 69]. It is suggested that some of the genes found in ColV plasmids are found to be involved in the development of infection in avian [70].

2.4.5 Capsule

APEC strains are classified into sero-groups such as O78, O18, O2, O1 and some of uncategorized strains based on the K1 capsular antigen. It was investigated that the three strains of APEC expressing KI capsule were found less resistance to other K antigen while high resistance to serum bactericidal effect [48, 71].

2.4.6 Other Virulence Factors

Beside the well-known virulence factor, APEC strains include pathogenicity islands, and the locus of enterocyte effacement [65, 72-74]. Some other genes recently found to be involve in virulence of APEC, *ibeA* and *kps*-MTII are linked with APEC virulence [65, 75]. Although *gimB, ibe* and *kps*-MTII were not found in most of APEC strains [74, 76] and cannot, therefore, one of the defining trait of APEC, may be significant minority trait having role in increase of6 APEC virulence.

2.5 Role of Curli Fimbriae in Pathogenicity

E. coli produces a numerus variety of adhesions that permit the microbe to persist, colonize and eventually leads to disease. *E. coli* synthesize long and rigid filaments known as pili or fimbriae that allow adhering the microorganism to a various types of cells [77, 78]. It is expected that different microorganisms produce adhesion more than one type and this adherence is one of the main causes that lead to bacterial pathogenesis. A thin and flexible aggregative filamentous protein mentioned as curli is frequently produce by *E. coli* have been found to related with laminin and fibronectin binding and observed as possibly erythrocytes hemagglutination of chicken [50, 79]. The prevalence of these adhesions among APEC strains and their relation with air sacculitis and colibacillosis is not reported yet. In the present study of curli gene presence in isolates of APEC strains and its role in the avian pathogenesis of colibacillosis is still not determined [79].

Curli fimbriae related genes (*csg* operon) had been almost present all isolate of APEC strains [40, 47]. In another study curli related genes were found about 90% positive of the total isolates of APEC strains [80, 81]. A similar study was conducted and it was found that isolates form respiratory tract with a disease of ostriches had been 70% positive for the curli associated sequences [82]. A study reported the presence of *csgA gene* sequence in all of the isolates of APEC strains from chicken with septicemia and in none of the isolates from intestinal *E. coli* isolates of microbiota of chicken. However, a similar study was conducted and it was found that all isolates of APEC strains from healthy and chicken with septicemia were positive for the *csgA* gene [82, 83].

2.6 Zoonosis of APEC

A well-known phenomenon is that; Antibiotic resistance strains of *E. coli* can transfer from the poultry source in a food chain. The *E. coli* extra-intestinal comprise of several virulence factors, leads to cause diseases in the internal organs. Thus, the APEC resistance factors may be able to transfer through food chain to human can cause inference with treatment of extra intestinal, and urinary tract infection. As mentioned above matter may have impact on treatment of enteric infections and salmonellosis. Thus, the incidence in the resistance profile changing of APEC should be stated and evaluated [84]. It was observed that APEC isolates with zoonotic risk was initially associated to the fact of some ExPEC of avian and human

have share some virulence genes and shown closely related phylogenic backgrounds [85]. Genome sequence of the O1:K1:H7 APEC strain showed high similarity to human NMEC and UPEC [86]. Remarkably, comparison of a numerus number of ExPEC from chicken and human disease for presence of virulence factors and phylogenetic background was performed. it was observed that most of the isolates were genetically distinct pathotype groups (NMEC, APEC, UPEC) and distinct features, the study observed a potentially zoonotic relation with a genotype cluster that include ExPEC that overlapping traits [87]. Certain genes associated with large transmissible plasmid of APEC are found in both UPEC and APEC [88]. A similar study further explained that Plasmid of UPEC showed similarity with APEC plasmid comprise of virulence factors (aerobactin, salmochelin, and sit operons) [74]. In addition, NMEC and APEC have common virulence genes of ColV plasmids [87] and pathogenicity of meningitis in rats and urinary infection in mice can be contributed through APEC plasmids in *E. coli* [89, 90].

An analysis of subtractive hybridization with *E. coli* K12 strain and APEC strains showed that, APEC DNA of specific regions have high homology with the human ExPEC strains and O157;H7 strain [91]. Another study isolate strains from chickens' trades products showed phylogenic background, virulence profiles and O antigen matching APEC-O1 genome and human ExPEC isolates [86, 92]. In general, APEC-O1 has been found to share the highest level of homology of nucleotides and protein with UTI89 strain, isolated from uncomplicated cystitis patient followed by CFT073 isolated form pyelonephritis patient and 536 strain (O6:K15:H31) isolated form complicated UTI patient [86]. As poultry meat, comprise of the greatest levels of overall *E. coli* contamination. It has been revealed that most of the *E. coli* strains isolated from poultry have been positive for multi-drug resistance genes then *E. coli* obtained from other meat [85]. The increase in globally consumption of poultry meat could contribute to the acquisition of resistance against antibiotics in ExPEC and the rise of ExPEC infection in humans [93]. International travels and trades have been noticed that they contribute to the spread of Infection worldwide [94]. So the infection-control measures are difficult to be implemented. Alternative strategies are developing to eliminated or prevent these infections of ExPEC need the full understanding of virulence and transfer of these virulence with respect to Zoonosis risk, there is also the need of regular epidemiological

surveillance of the antibiotic resistance pattern of these bacteria both poultry and humans as well as other animals. The zoonotic risk route of bacteria transmission is depicted in figure 2.

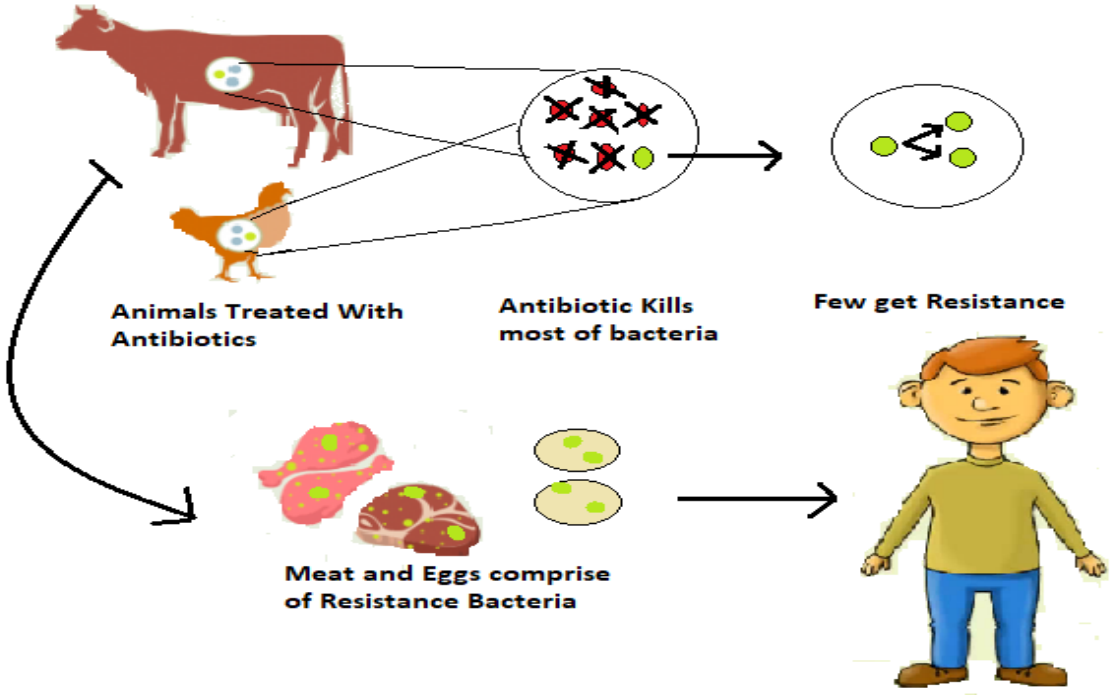

Figure 2: Zoonosis and antibiotic resistance bacteria transmission within food chain

2.7 APEC Infections and Economic loss:

Most of *E. coli* strains are considered as safe nonpathogenic, while some of their strains are responsible for serious infections and leads to several sever diseases. *E. coli* targets avian species and responsible for septicemia, respiratory tract infection and soft tissue infection. Major infections found in avian caused by *E. coli* were cellulitis, colibacillosis and sacculitis, results high mortality and morbidity rate in poultry, leads to huge economic losses to poultry industries [43, 62]. Other reported extra-intestinal infections caused by APEC include, omphalitis in embryos, salpingitis in laying hens and other respiratory tract infections. It is reported that the extraintestinal infection, which is most prevalent in broiler chickens is cellulitis by APEC, characterized by the presence of subcutaneous fibrin necrotic plaques and overlying chicken skin inflammation, leads to rejection of part or all of the corpses at processing. Extraintestinal infections by APEC include salpingitis in laying hens, omphalitis in embryos, cellulitis respiratory tract infections. The most prevalent extraintestinal infection is cellulitis found in broiler chicken caused by APEC and characterized by inflammation of

the overlying chicken skin and subcutaneous fibrin necrotic plaques presence, leads to denial of all or part of the corpses at processing [95]. The most important disease in broiler chicken caused by APEC strain is coli-septicemia.

Respiratory infection following to viral infection caused by APEC, is considered to be preliminary step for coli septicemia expansion in birds [62]. This infection typically occurs within 2 to 12 weeks' age of birds, also referred to as aero-sac disease. Majority of the aero-sac disease cases occur within 4 to 9 weeks of ages with a 20% mortality rate [42]. The respiratory tract infection usually followed by coli septicemia is frequently caused by the predominant APEC serotypes O78:K80, O1:K1 and O2:K1 [43].

Among the poultry diseases, Colibacillosis is the major disease causes by APEC with a high morbidity and mortality in poultry industry, causes high economic losses. Similarly, other diseases like peritonitis, synovitis salpingitis, pericarditis, air sacculitis, yolk sac infections or osteomyelitis can induce by APEC strain [96, 97]. In hatchers and broiler chickens, a syndrome name swollen head syndrome caused by APEC strains has caused substantial losses in the poultry industry, responsible for mortality rate of 3 to 4% and 2 to 3% of the birds and egg production respectively [98]. The antibiotic therapy is the primary treatment against APEC infection, to reduce the morbidity and mortality. The antimicrobials need to be carefully used in order to preserve the usefulness of therapeutics in both humans and animals, since the indiscriminate use of antibiotics results to the selection of resistant isolates [42].

2.8 Current Treatments of APEC Infections

2.8.1 Antibiotics and their Combination Therapy

Antimicrobials are used as a prophylaxis and therapy to controlled infection and diseases [99, 100]. *E. coli* is one of the main cause of infections such as nosocomial urinary tract and community-acquired infections of about 50 and 90% respectively [101]. The most frequently prescribes drugs are quinolone and β-lactam antibiotics used for the treatment in clinical settings [102]. Sever infection and gram-negative sepsis caused by Pseudomonas spp. are also treated through combination therapy, which usually comprise of an aminoglycoside and broad-spectrum β-lactam or β-lactam and Fluoroquinolone. However, a combination of colistin usage has increased for the multidrug resistance strains as a last resort treatment [103-109].

High resistance has found in the *in vitro* antibiotic susceptibility pattern to frequently used antibiotics such as nalidixic acid, ampicilin, penicillin, amoxicillin, cephalexin, ciprofloxacin and erythromycin [99, 103, 105]. Previous studies observed successful combination therapies that include a carbapenem, colistin, or rifampin, an aminoglycoside, ampicillin/sulbactam [110-112]. It is reported that for carbapenemase-producing Enterobacteriaceae, colistin-tigecycline and other antibiotic combinations include a carbapenem, an aminoglycoside, fosfomycin, colistin, rifampin or tigecycline have been found advocate [113-116].

Antibiotics resistance, a main challenge through worldwide and their alternative solution is urgently needed [2, 117]. Antibiotics are used worldwide for the treatment of different microbial infection. However, the resistance against antibiotics increases day-by-day [118]. Some reports showed that, in USA at least 2 million people is infected by antibiotic resistance bacteria and at least 23000 are dead annually by these infections and some death were recorded for the other conditions caused by these antibiotic resistance infection [119, 120]. To develop new drugs, it is not commercially feasible if there is any high chance of becoming uselessness after introduction of new drug to the market [121]. One of the main shortcoming is the failure to discover entirely new anti-microbial agent, and the present antibiotics now been altered from those discovered over the last few decades, to produce new standard form [122]. This is an opposition to spending more money on research & development. the alternatives for conventional antibiotics is needed because of the emergence of microbial infection diseases [123]. Antibiotics against Salmonella spp. found in industries of poultry, were used to decrease their level at each and every phase of the production in farms. Still Salmonella spp. considered as a main cause of foodborne disease worldwide and main reservoir for this zoonotic pathogen is considered are chickens [124, 125].

2.8.2 Vaccines

APEC strains have high diversity, which limits the use and efficacy of vaccination, which cannot be use for enormous scale properly. Several vaccines have tested against APEC based on attenuated/killed, experimentally. They give less efficient Protection against heterologous strains (such as APEC), but in general, the protection against homologous strains infection is sufficient [42]. However, it is reported that certain level of protection against heterologous strains has obtained with an attenuated vaccine [126]. Young birds that are compromised with

homologous strains were treated with passive immunization via breeder hens is effective up to 14 days [127]. A good homologous protection was detected against APEC possessing the same fimbriae, through vaccine against virulence factor like fimbriae [128].

Poulvac® *E. coli* is a live attenuated vaccine with a knockout of *aroA* gene of *E. coli* strain EC34195 (Fig.3). The gene is responsible for the biosynthesis of aromatic amino acid produced in the target strain APEC O78:K80 [129]. The genetic modified strain is named *aroA*- PTA-5094. This attenuated vaccine is capable of triggering a passive immunity in poultry against pathogenic and wild *E. coli* and their infection. The deletion of *aroA* gene make the *E. coli* non-virulent and unable to form a self-survival population of bacteria since the mutant strain has lost the ability to produce the amino acids, basic for its survival [130].

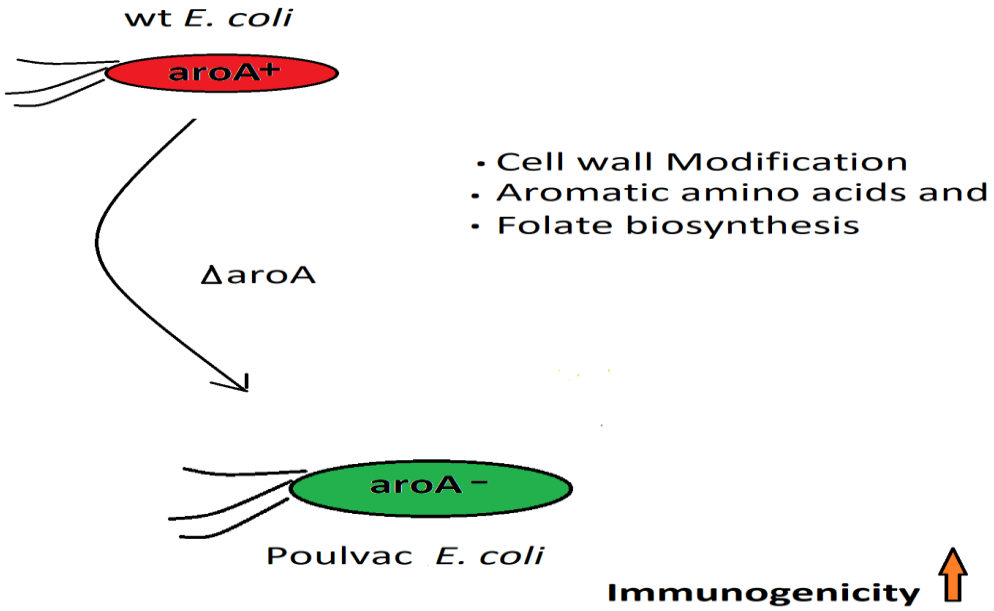

Figure 3: Vaccine against APEC-O78 with *aroA* knockout of wild type *E. coli* known as Poulvac *E. coli*

2.8.3 Bacteriophage Therapy

The bacteriophages are recognized to have greater benefits linked with the phage therapy over the use of different antibiotics [131-133]. Phage therapy is an alternative method to combat against pathogens and to reduce the use of synthetic antibiotics [134]. The first study was conducted for the control clinical trials of the therapeutic phages preparation since 2009. It was reported a higher safety as well as efficacy for chronic otitis against multi drug resistance

P. aeruginosa [135]. Recent study reported that the phages have been recommended to be involved in a nebulizer to cure cystic fibrosis patients as well as bacterial infection in lungs [136] and could be used for the treatment of pulmonary infection by spraying as dried phages in respire-able powders [137].

It was reported that the administration of phage therapy with mixture of phages called cocktail into ears kill *P. aeruginosa* without obvious toxicity and become a potential, effective and appropriate treatment for the *P. aeruginosa* infection [138]. Oral delivery of phages such as adding to drinking water or feed and direct administration as a capsule has no such good results over the intramuscular injection and aerosol spraying [139]. Some studies suggested that the low efficiency of oral administration of phages is limited by high pH at gastric, stop the spread of phages [140].

A study was conducted to reduce the Salmonella colonization and it was found that bacteriophages were highly active against serotypes of *Salmonella enterica, Typhimurium, enteritidis* and Hadar [141, 142]. Several other studies were also done using phages against pathogens found in broiler chicken such as campylobacter and Salmonella spp. concentration to reduce their colonization [143-145].

Still an appropriate administration targeting specific bacteria is required for veterinary therapy application, including a comprehensive methodology through feasible strategy, the phage-host interaction details, consideration of all physical and chemical dynamics, and dose optimization [146]. In previous studies, bacteriophages are used against zoonotic pathogens on food surface as well as live animals [141].

Colibacillosis is the commonest infectious disease of farmed poultry, was treated by using bacteriophage and initial studies demonstrated that the mortality was reduced from 85 to 35%, furthermore when the amount of bacteriophage was increased the poultry birds were completely protected [147]. Pathogenic *E. coil* was experimentally administrated to cause diarrhea in calves and piglet, after 8following the bacteriophage administration the diarrhea was cured [148, 149]. Recent studies showed that the phage therapy have great potential against bacterial pathogens. Such as, the intraperitoneal injection of (vancomycin-resistant *Enterococcus faeicum* or *S. aureus*) causes death in mice but the mice were saved by the administration of an intraperitoneal injection of bacteriophages after initial injection. Results

showed that the harmful effect of bacteria was significantly reduced [150-152]. While it is obvious that, in some of the former Soviet Union countries and Poland, phage therapy has been practiced for many decades, but there are still many doubts related to its competence to switch the antibiotics [153, 154]. Since phages are still not suggested for final consideration for a magic bullet and might not applicable in certain circumstances [155]. A major limitation of phage therapy is the emergence of bacteriophage-insensitive mutants (BIMs) that has long been perceived [156]. Still unlike antibiotics, phages continuously advance to avoid their host defense and resistance bacteria are mostly less suitable or less infectious than their phage sensitivity counterparts [142, 157]. Phages are found as a carrier of toxin genes or virulence factors, so full knowledge of phage genome sequence would be helpful to estimate the possible difficulties in phage therapy [158-160]. Some experimental approaches based on the detection of shiga toxin (*stx-2*) gene by a phage enrichment culture followed by PCR were used and it was found that bacteriophages carrying shiga toxin (*stx2*) gene infecting and transferring the gene to *E. coli* O157:H7 [161]. In previous study, it has reported that the phage pp01 was used against *E. coli* O157:H7. A rapid decrease in viable cell count was observed followed by the emergence of phage resistant bacteria and the resistance bacteria were found, have changed *OmpC* expression. It was proposing that this protein is involves in the phage attachment and it is found in the previous study that *OmpC* to be used by phage receptor during attachment to *E. coli* O157:H7 [162, 163]. Another study reported that phage resistance for most kind of bacteria, easily arises in susceptible *in vitro* culture and for *Campylobacter jejuni*, it has been observed to arise resistance against phages *in vivo* in poultry [164]. However, some mutant phages were also identified, with an altered host range, which can overcome the phage resistant bacteria [165].

In competition with bacteriophages, virophages takeover DNA of a virus in command to replicate and frequently distort virus/phage particles in aquatic environment, making viruses less virulent with respect to infecting their host. One of the main problem in phage therapy is to maintain the phages survival and persistence on different surface, because of influence of some external forces on a phage-host interaction within the surrounding environments [146, 166].

2.8.4 Regulation of Virulence Gene Expression:

2.8.4.1 ncRNAs

Gene expression regulation through small RNAs has been observed in *E. coli*, many years ago since unexpected discovery of MicF sRNA. MicF is involved in regulation specifically down regulation of *ompF* expression, which encodes major porin of outer membrane, *OmpF* [167]. There were certain RNAs such as OxyS and DsrA were found incidentally [168-170] having similar characteristics with the identification of several sRNAs using systematic searches [169, 171, 172], which showed the involvement of sRNA widely in regulation of gene expression, predominantly at level of post-transcription. However, still the biological function of many new identified sRNAs are not yet elucidated completely, but the function of major class sRNA of *E. coli* that bind to Hfq [173], a RNA chaperone and act by the imperfect base pairing lead to negative regulation of stability and translation of target mRNAs under definite stress condition.

The ncRNAs have been found in all species of bacteria examined to date. All species of bacteria have been positive for ncRNAs tested up to date. In species specifically, *S. enterica, E. coli, S. aureus,* and some other species which have been found positive for sRNA. Investigated in detail and majority of small RNAs involves in post transcription regulation of main metabolic processing, which includes adaptation to nutritional processes, various stress responses, biofilm formation, quorum sensing, motility and pathogenicity [174-178].

The ncRNAs are about 50-300 nucleotides transcripts, act as a regulator of messenger RNA and protein expression, by hindering translation and altering stability [179]. Hundreds of ncRNAs have been found all over bacteria naturally, mostly assisted by high throughput sequence technologies and computational tools [180-182]. Over hundreds of ncRNAs have been confirmed in *E. coli* alone. Many of them are known to have to regulate expression of multiple mRNA targets, several of them are identified genetically and biochemically but still others are not yet covered. Notably, a several significant ncRNAs specifically in gram-positive, depend on the Hfq protein (RNA binding protein) as a cofactor, which has the ability to enable mRNA targeting [183, 184].

2.8.4.2 Hfq Protein

Hfq was first discovered in *E. coli*, it was one of the first protein, known member of an extensive RNA-binding protein family, which can be almost found in every cellular organism form all the three domains of life [185]. Several mechanisms of Hfq-mediated regulation have been reported at the level of RNA stability and translation [173, 175, 186-189]. It was assume that Hfq is provided by bacteriophage but the first evidence was reported to the potential impact of Hfq could provide to the bacterium itself since 1990s. Hfq loss showed the reduction in fitness and stress response impairment to diminish the virulence in *Brucella abortus* a pathogenic bacterium [190, 191]. It was also reported that Hfq also regulates turnover or translation of numerous cellular mRNAs [192, 193].

Bioinformatics and structural studies conducted a decade ago, reported that Hfq is part of the Sm family, explaining that Hfq belong to origins date back to the last common bacterial, eukaryotic and archaeal lineages ancestors [173, 194]. It was also become clear that Hfq linked with the regulatory sRNAs to stimulate the sRNAs base pairing with the related target mRNAs [194-196]. The lifetime and translation of the target transcript is affected by the sRNA-mRNA pairing. The Hfq connection work as facilitator of the trans-actions pf sRNAs could reason for several complex phenotypic effects that are reported in the gene knockout studies conducted earlier [190, 197]. It is predicted that Hfq have been present in at least 50% in all bacterial species [190].

2.8.4.3 RNase Endoribonuclease

RNase E is considered as the dominant catalyst of overall mRNA translation in γ-proteobacteria and known factor in target mRNAs decay, induced by sRNAs [198-204]. It is also reported that RNase E was found to degrades unpaired sRNAs (called to be coupled degradation) as well as paired with targets (mRNAs) [201]. The major endonuclease RNase E able to degrade or processes stable RNAs and mRNAs. During processing it form RNA degradosome, a multi-protein complex, incorporating RhiB RNA helicase, and enolase and polynucleotide phosphorylase through its C-terminal scaffold region [205, 206]. It is believed that RNA degradation act as common RNA decay machine and its components of the complex assist during the degradation of many RNAs. The RNase E truncated at C-terminal in mutants, which disable to form degradosome led to slow the coupled degradation of sRNAs

and mRNAs [201, 202]. It is suggested that either the C-terminal scaffold region of RNase E and/or degradosome is necessary for an optimal and efficient degradation of both RNAs (sRNAs and mRNAs).

2.9 Mechanism of Action of sRNA

2.9.1 Gene Regulation

Notably, a large number of ncRNAs specifically in gram negative bacteria is assist by RNA binding protein Hfq, as a cofactor to target mRNAs (see Fig.4) [167, 207]. Homologs of Hfq are extensively found across bacterial species but there are still many species which lack these RNA binding proteins [208]. Most of sRNAs repress trans-encoded target mRNA by lack of perfect and limited base pairing [18, 170, 187, 209]. Hfq a RNA chaperon is not just necessary for RNAs regulation as target annealing [189, 194, 210, 211] but also for sRNAs protection from nuclease degradation [201, 212, 213]. The natural sRNAs bind to the ribosomal-binding site (RBS) which is found in the 5′ UTR of mRNA, sequester the RBS to stop loading of 30s ribosome. First model of sRNA action at the level of translation initiation was proposed for MicF-ompF in *E. coli* [167]. This model of was supported by many examples of sRNAs, paired around the shine-Dalgarno sequence or start codon AGU, to inhibit translation *in vitro* successfully [207, 208, 214-217]. It was observed the target gene expression could be suppressed without destruction of associated mRNA *in vivo* [194, 218]. Following the gene repression a translational level, interacting RNAs (sRNA-mRNA complex) are often mediated and utilized as substrate by RNase E an endonuclease also known as RNase III [198, 201, 215, 216]. Specifically, RNase E that act on bulk of mRNAs of enterobacterial [24, 219-221] and prefers AU-rich single stranded RNA, which has been found involved in destabilization of sRNA-mediated mRNA in *E. coli* [198, 199, 201, 222]. A study suggested that Hfq-bounded sRNA leads to recruit RNase E actively to target mRNA [222]. However, it has been reported that translation initiation inhibition in sRNA-mediated silencing of a gene is still considered as the crucial event, by the discovery of 5’UTR targeting in the upstream [223-225]. In contrast, it reported that the role of sRNA and target mRNA decay was thought to make robust and irreversible regulation [170, 201, 202]

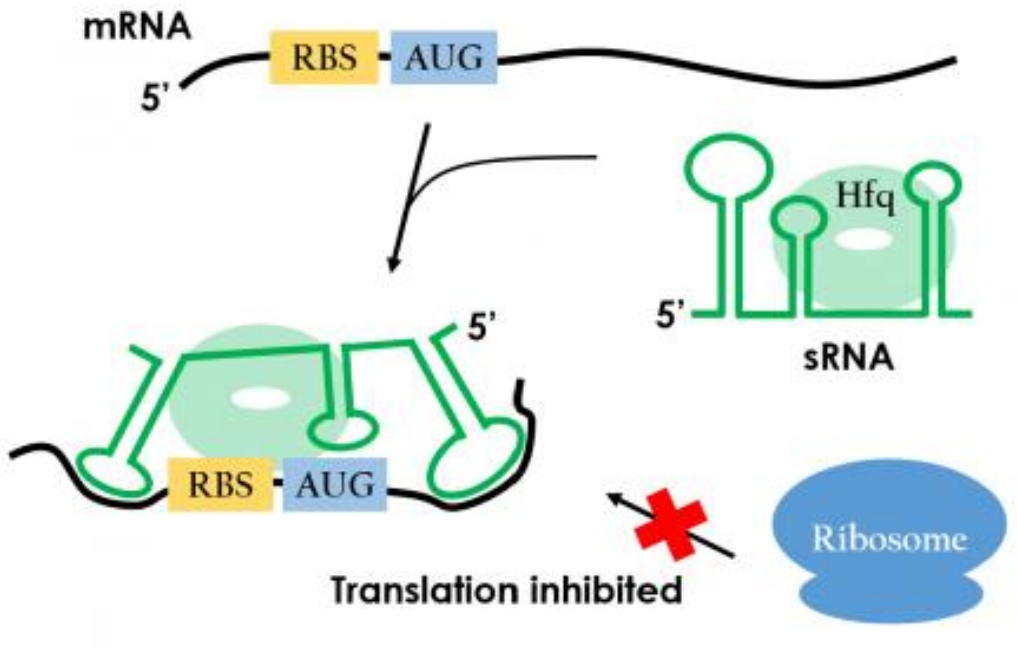

Figure 4: Hfq mediated sRNA silencing of Target mRNA by inhibiting Ribosomal attachment to mRNA [226]

2.9.2 Synthetic sRNA Designing

Synthetic sRNAs were constructed to achieve the gene regulation at mRNA level in *E. coli* [227, 228], after the discovery of antisense ncRNAs complementary to mRNA, involved in translational regulation [229]. It was also reported that synthetic sRNAs were constructed for many other bacterial species such as Staphylococcus spp. [230] Bacillus spp. [231], Mycobacterium spp. [232, 233] Lactobacillus spp.[234] and Clostridium spp.[235].

However, initially the efficiencies of sRNAs were observed very low and mostly gene-dependent, the potential of antisense RNAs usage to silenced target gene has driven a new variety of sRNAs design, to efficiently express these antisense-binding sequences (sRNAs) [236]. For efficient expression of trans-regulatory sRNAs, a basic modular design has been engineered which includes a promoter, binding domain of antisense sRNA, Hfq binding domain or a scaffold for stability followed by a terminator (see Fig.5) [237].

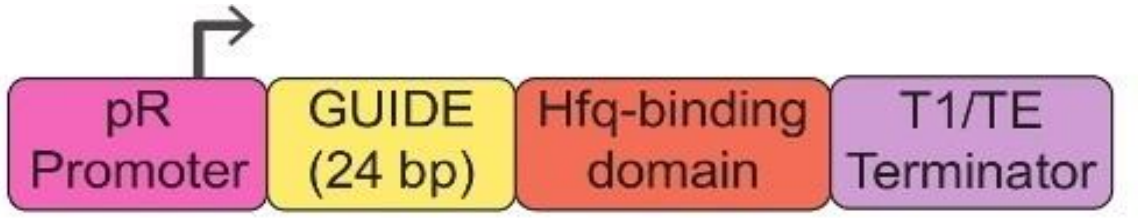

Figure 5: Expression cassette for higher silencing efficiency of a target mRNA

2.10 Expression System of sRNA:

In vivo expression of a synthetic ncRNA, it is usually inserted in a plasmid with a promotor region upstream of the ncRNA to enable the transcription initiation. It is has been observed that effective inhibition of target mRNA is strictly depend on appropriate promotor selection i.e. tightly regulated promotors [237-239]. Regulatory promoters with temperature or ligand inputs are particularly convenient as the expression can be turned on and off easily [240, 241]. Some of recent studies have employed a range of promoters. Significantly, the transcription starts site (TSS) of the particular promoter should be identified. Among studies at least one study selected and tested promoters of different types such as PTet, PT7 and PTrc, for efficient silencing of gene conferring to the target mRNA sensitivity [238].

2.11 Delivery System of sRNA:

A delivery system was developed to carry sRNA and expressed efficiently within the target host. Expression constructs of the sRNA from a DNA vector (phagemid) expressed in *E. coli* first delivered to living cells using the modified M13 phage helper system. The phagemid is a plasmid with f1 origin of replication, derived from a phage. The delivery vector is M13 helper phages, produces by a helper plasmid M13KO7, carries all the required machinery for viral particles production, but itself not able to packing and replication. The co-transformation of phagemid and M13 helper plasmids into *E. coli* is done. Phagemid is replicated alone at f1 origin, packaged in M13 helper phage and finally secreted out of the cell without harming or bursting host cell. The vectorized M13 packaged phagemid infect *E. coli* via specific Pili known as F pilus. The schematic representation of delivery system is given in figure 6 [20, 242-244].

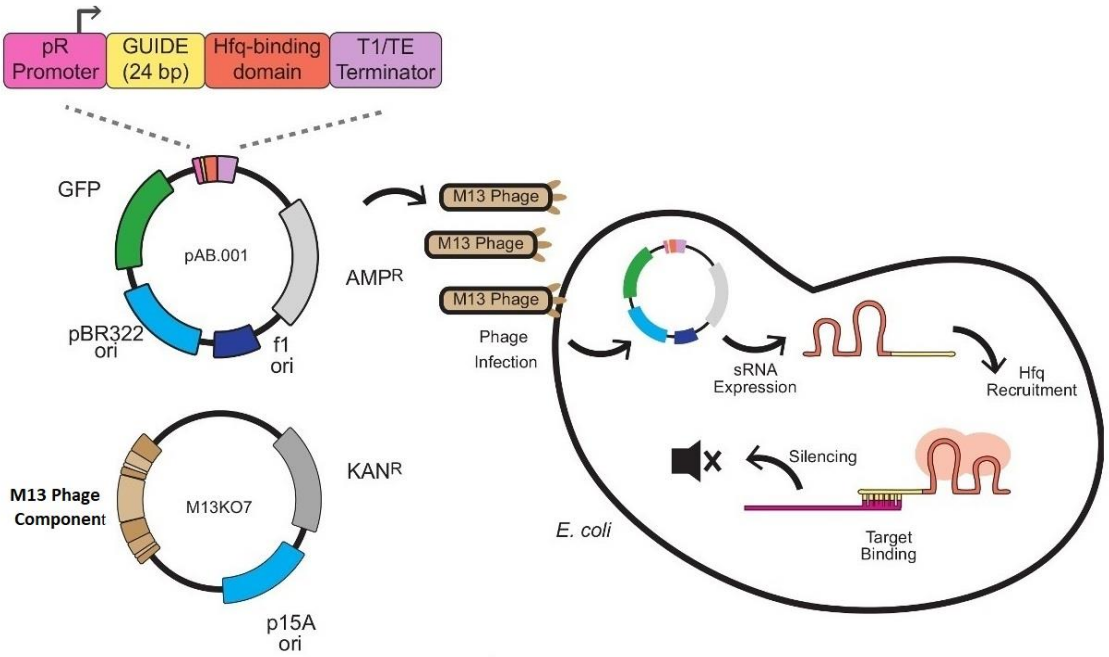

Figure 6: Gene knockdown in *E. coli* with sRNA expression cassettes delivered by M13 helper phage, illustrated by Bernheim et al., [20]

Chapter 3:

3 Materials and Methods

3.1 Materials

3.1.1 Equipment

All the equipment used in this study is given in Table.1

Table 1: Equipment used in this study

Equipment	Company
ELISA 96 well Plate Reader	
Gel Electrophoresis Tank,	WEAL Tech
Gel Doc	Dolphin-DOC
Gradient PCR	Applied Biosystems
Hot Plat	Eppendorf
Incubator	Memert
Incubator shaking	JSR
Laminar Air flow cabinet	Technico Synthetic supply
MyiQ RT-PCR	BIO-RAD
Nano-Drop	Colibri
PCR	Applied Biosystems
pH meter	InoLab
Refrigerated Centrifuge	HERMLE
Sterile Filter 0.22um	Sartorious
Table top centrifuge	Sigma
Table top spectrophotometer	Optima
Water bath	Memmert
Vacuum Concentrator Plus	Eppendorf
Vortex	Heidolph

3.1.2 Chemicals

The chemicals used in this study are given in Table.2

Table 2: Chemicals used in this study

Chemicals	Company
Acetic Acid	Sigma-ALDRICH
Agarose	MERCK USA
Calcium Chloride	Scharlau Spain
Chloroform	DAEJUNG China
EDTA	ROTH
Ethidium Bromide	Invitrogen
Ethanol	MERCK USA
Glucose	Riedel-deHasen Germany
Glycerol	MERCK USA
Glacial Acetic Acid	Scharlau Spain
Hydrogen Chloride (HCL)	Scharlau Spain
Isopropanol	EMPLURA Germany
Magnesium Chloride ($MgCL_2$)	Scharlau Spain
Potassium Acetate	MERCK USA
Sodium Chloride (NaCl)	Scharlau Spain
Sodium Hydroxide (NaOH)	Scharlau Spain
Sodium Dodecyl Sulfate (SDS)	Phytotechnbology Lab
Tris-Base	Phytotechnbology Lab
Tris-HCL	Phytotechnbology Lab
Trypton	OXID

3.1.3 Culturing Media

All the culturing media used in this study are given in Table.3.

Table 3: Media used in this study

Media	Company
LB Broth: Trypton(1%), NaCl(1%), Yeast Extract (0.5%)	OXID
Nutrient Agar	OXID
Trypton Soy Broth	OXID
Brain Heart Infusion Media	OXID

3.1.4 Bacterial Strains

All the strains used in this study are given in Table.4

Table 4: Bacterial strains used in this study

Strains	Provided by
E .coli DH5α	Dr. Fazal Adnan ASAB Labs
APEC-O1	Dr. Mashkoor Galani (Agriculture Uni Faisalabad)

3.1.5 Plasmids

All the plasmids used in this study are given in Table.5

Table 5: Plasmid used in this study

Plasmids	Provided by
pAB.001	Bernheim et al., 2016
M13KO7	Bernheim et al., 2016

3.2 Methods

3.3 *In silico* Approach

3.3.1 Primers Designing

Primers were designed for *csgA* gene detection using the primer-blast an online tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design primers[245]. The *csgA* gene was first retrieved from NCBI (https://www.ncbi.nlm.nih.gov/nuccore-/NC_000913.3?report=fasta&from=1104447&to=1104902) and EcoCyc (https://ecocyc.org/ECOLI/sequence?type=GENE&object=EG11489) databases, the FASTA sequence was entered in the primer-blast interface and search for suitable primers [246]. The suitable primers were selected and ordered (see Table.1).

3.3.2 Designing of GUIDE Sequence and sRNA Expression Cassettes:

The *csgA* gene complete sequenced was retrieved from the EcoCyc *E. coli* database (https://ecocyc.org/ECOLI/sequence?type=GENE&object=EG11489). Designing sRNA for the target gene the coding sequence, 24bps was selected from the +1 to +24 position beginning from the state codon. It is observed that knockdown is less efficient other than first 24 nucleotides of the target gene [240]. To produce the GUID sequence the reverse complement of the TARGET sequence was made for the sRNA cassette (See table 7). Expression cassettes was composed of 292bp and consist of the pR promoter, GUIDE sRNA sequence, Hfq binding domain and T1/TE transcriptional termination sequences, arranged in sequence (see table 8). To facilitate cloning additional cloning sites were added to sRNA expression cassette into the target pAB.001 vector. The complete sRNA cassette integrated in the pAB.001 phagemid vector with a functional f1 replication origin were kindly provided by Bernheim et al.,[20, 247].

3.3.3 Binding Energy off Targets and sRNA Structure Prediction

The binding energy was predicted using IntaRNA tool (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp), to find out the sRNA and mRNA interaction [248]. The off target was predicted using CopraRNA tool [248], search limited to the host genome (http://rna.informatik.uni-freiburg.de/CopraRNA/Input.jsp). Similarly, the secondary structure of sRNA with scaffold sequence were also predicted using RNAfold tool (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi), to find out the compatibility of

sRNA with Hfq protein, the sRNA structure must have two or more stem loops for efficient interaction with Hfq protein [249, 250].

3.4 *In vitro* Approach:

3.4.1 DNA Extraction

Bacterial strain APEC-O1 was inoculated in a 10ml LB media overnight at 37°C with shaking. The pellet was obtained by centrifugation at 8000rpm for 5min. The pellet was re-suspended in TE buffer and incubated at 95°C for half an hour, followed by centrifugation 10000rpm for 10min. the supernatant was discard and then DNA was remained as a pellet were added by 50µl of TE and stored at -21°C for further use [251, 252].

3.4.2 Gene Detection through PCR:

DNA was extracted from APEC-O1 using the protocol mentioned above. For gene detection the PCR was performed to amplify the target gene, using commercial kit. DNA of the APEC-O1 of about 5µl was mixed with the 25µl of pre-aliquoted DreamTaq Green PCR Master Mix (Thermofisher Scientific) with addition of 1µl of each reverse and forward primers and raised the volume up to 50µl with Nuclease free water, according to the manufacturer's instructions with certain modification (see table 10). For the PCR the Applied Biosystem 2720 thermal cycler was use. The condition was applied as per DreamTaq Green PCR Master Mix manufacturer's instructions with certain minor changes (see table 11). The amplified gene was run on the Agarose gel and their amplicon size was confirmed by gel band size compare with 1 kb ladder, on the Gel-Doc viewer [253, 254].

3.4.3 GUIDE Sequence and Site-directed Mutagenesis:

The 24bp GUIDE sequence in the current sRNA expression cassette against *csgA* gene was obtained. The reverse and forward primers comprise of about 18-20 bps complementary to the prevailing sRNA cassette which flanks the new designed 24bp long GUIDE sequence was designing manually [20, 240, 255]. Primers were obtained from the commercial oligonucleotide synthesis comprise of 62 bps long sequences (see table 9). PCR reaction mixture was prepared using template of the phagemid and Phusion polymerase (see table 12). Finally, PCR conditions were use as recommended by polymerase supplier (see table 13).

3.4.4 Chemically Competent Cells

Competent cells of *E. coli DH5α* were chemically produced by using the following protocol. The cells from cryo-stock were first streak in nutrient agar plates using inoculating wire loop and incubated at 37°C overnight. A 10ml fresh LB broth were inoculated with single colony and kept overnight at 37°C followed by 50ml fresh culture inoculated with 1ml of overnight culture and incubated for about 2hr at 37°C, till OD600 rise to 0.5-0.6. Culture was transfer to chilled 50ml falcon tubes over ice and incubates for 10min then centrifuge at 4000rpm for 10min at 4°C. Supernatant was discarded and the pellet was re-suspended in 10min of chilled Solution I (see table 15) and incubated for 30min over ice. The cell suspension was centrifuge again at 4000rpm for 10min at 4°C. Pellet was re-suspended in 1ml of chilled Solution II (see table 15). The re-suspended pellet was transfer to chilled Eppendorf tubes of about 50µl each followed by treated with liquid nitrogen before storing at -80°C [256, 257].

3.4.5 Heat Shock Transformation

Plasmid pAB.001, comprised of sRNA expression cassette for the target gene were transformed into chemically produced competent *E. coli* DH5α cells using the following protocol. About 5µl of plasmid was added to thawed competent cells on ice of about 50ul, mix with pipetting on ice and incubated for 30min followed by heat shock for 2min at 42°C in water-bath (45sec sometime). After heat-shock the cells were directly transfer to ice and incubated for 2min. About 1ml of fresh LB broth was added to the cells and incubated at shaking incubator for 2hr at 37°C. LB agar plates were prepared using recommended quantity of each Antibiotics (see table 16). After 2hr' incubation the of the Transformed cell, culture was centrifuge at 300rmp for 5min and the removed the 2/3 of supernatant, mixed well the pellet and spread the cells culture of about 100-150µl on each Antibiotic LB agar plates, incubated overnight at 37°C. Colonies were confirmed by extraction of plasmid and confirmed their band size on agarose gel. The plasmids were further verified through sequencing [256, 258-260].

3.4.6 *E. coli* DH5α Cloned Phagemid Glycerol Stocks

The *E. coli* DH5α cloned with sRNA expression phagemid sequenced verified was inoculated in a 10ml culture. Glycerol stock of the *E. coli* DH5α Cloned sequence verified was prepared using the following protocol. Overnight culture *E. coli* DH5α clone was centrifuge at

5000rpm for 5min, the pellet was to 40% of glycerol and 1ml LB media in a screw cap cry-tube. The stock was stored at -80°C until further use. The remaining culture of was used for the sRNA expression phagemid [261, 262].

3.4.7 Plasmid DNA (M13KO7 and pAB00.1) Extraction

Plasmid DNA from cells was isolated according to the alkaline lysis protocol [4]. Cells contained Plasmid was inoculated in a 25ml culture with suitable antibiotics. Pellet was obtained from overnight culture at 8000rpm for 5min using Eppendorf tubes. Chilled 250µl of Resuspension buffer (see table14), was added and mixed well using vortex, about 10µl of RNase A was added and incubated for 5min on top of a bench. Then Lysis buffer (see table 14) was added of about 300µl and gently invert the tubes several times and incubated on top of the bench for 5min, this will break the cells. Followed by adding chilled Precipitation buffer (see table 14) and gently inverted the tubes several times and incubate on ice for 10min, which precipitate all the cell debris and large threads of DNA. The mixture was then centrifuged at 13000rpm for 10min at 4°C, the supernatant was transfer new Eppendorf tubes and chilled isopropanol absolute was added about 1:1 to the supernatant. Incubate on ice for 5min and centrifuged at 13000rpm for 15min at 4°C. a white pellet was obtained and the supernatant was discard. 200µl of 80% Ethanol was added to wash the pellet by centrifugation at 13000rpm at 4°C for 10min. The pellet was air dried and 50µl of nuclease free water was added and stored at -21°C for further use [262-264].

3.4.8 Production and Harvest of M13-packaged Phagemid Stocks

Both the sRNA expression phagemid and M13 helper plasmid using alkaline lysis method previously described [264]. Similarly, chemically competent *E. coli* DH5α cells are produce as mentioned earlier. Sequential transformations with 5µl of first M13KO7 helper plasmid followed by transformation of sRNA expression phagemid and co-transformed were then selected using LB agar plates with specific antibiotics for both plasmids (kanamycin for M13KO7 helper plasmid and ampicillin for sRNA expression phagemid/pAB.001). A single colony of the co-transformed *E. coli DH5α* was culture further in a 20ml LB media with selective antibiotics overnight at 37°C using shaking incubator. The overnight culture was centrifuge at 3300g at room temperature for 10min and the supernatant was collected in a new 14ml falcon tube and filtered through sterile filter (0.2µm). The filtered M13 packaged

phagemid was stored at 4°C for further infection. The filtrate can easily preserve for few weeks without loss of proper activity [20].

3.4.9 APEC Infection with Packaged Phagemids

A single colony of APEC-O1 strain was inoculated in a 10ml culture LB media with appropriate antibiotics overnight at 37°C with shaking. The overnight cultures of APEC-O1 was diluted in a 5ml media at 1:100 and incubated at 37°C with shaking, about 2hr till the 0.3OD at 600nm was obtained using bench top spectrophotometer. The target cells were infected by adding M13 packaged phagemids with a ratio of 1:100 by volume to achieve the highest infection rate of target population about 99%. The infection was remained about 1hr at 37°C using shaking incubator [20]. Glycerol stock was prepared using the protocol used earlier for further ncRNA expression analysis.

3.4.10 RNA Extraction

A single colony of APEC-O1 was inoculated in a 10ml culture LB media with appropriate antibiotics overnight at 37°C with shaking. The overnight cultures of APEC-O1 was diluted in a 20ml media at 1:100 and incubated at 37°C with shaking, about 2-3 hr until the 0.6 OD at 600nm was obtained using bench top spectrophotometer. The pellet was obtained through centrifugation at 8000rpm for 5min, the supernatant was discarded and the Trizol was added about 700µl per 3ml culture media. The pellet was mixed using vortex at incubated on ice for 10-15min, followed by adding 0.2ml of chilled chloroform per 1ml Trizol and mixed well using vortex. The mixture was then centrifuged at 10,000rpm at 4°C for 15min. Centrifugation separated the mixture into three phases, a red organic phase that contains the protein, an interphase, frequently white that contains the DNA, and a colorless upper aqueous phase that contains RNA. The colorless upper aqueous phase was transfer to a new clean tube, using pipette of 200-µl range and avoided the interphase, which mostly contains DNA. The colorless phase was usually up to 700µl could be easily transferred into new tube. Total RNA was precipitated by adding 0.5ml of isopropanol per 1ml Trizol used earlier; the sample was mixed gently by inverting 5X and incubates on ice for 5min followed by centrifugation at 10,000rpm at 4°C for 10min. The supernatant was removed and the RNA pellet was washed by adding 1ml 75% Ethanol per 1ml Trizol used earlier and mixed gently by inverting the samples several times. The sample was centrifuged at 14000rpm for 5min at 4°C. The

supernatant was removed and tube was inverted on a clean tissue Kim-wipe. Pellet was dried and incubated at 55°C for 5min and finally, the pellet was re-suspended in 50µl of DEPC treated water [265].

3.4.11 Expression Analysis through Real time PCR

Overnight cultures of *E. coli* were diluted 1:100 into fresh LB. The bacteria were grown to the logarithmic phase, and RNA was isolated through Trizol method. Contaminating DNA was removed from the samples with RNase-free DNase I. With specific primers (see table 1), Cybergreen Master Mix was used, the temperature condition mixture protocol was followed as mentioned by manufacturer. The data was normalized to the house keeping gene *uida* transcript. Using the threshold cycle ($\Delta\Delta CT$) method by Livak et al., , the relative fold change was calculated [266, 267].

3.4.12 Biofilm Assay

Effect of the knockdown of *csgA* gene through sRNA on biofilm formation was assessed using crystal violet (CV) assay [268]. Single colony of APEC-O1 and silenced APEC-O1 were grown in LB broth overnight at 37°C with shaking, the overnight each cultures was diluted on Trypton Soya broth (TSB) fresh media with ratio of 1:10, the 0.3OD at 600nm was obtained by 2hr' incubation at 37°C with shaking. The fresh each cultures were further diluted at ratio of 1:10 in fresh TSB media as a final culture dilution for the final testing. The biofilm assay was performed in 96 well plate adding 200µl final culture dilution of each strain in the study. Followed by incubation of plate without shaking at 37°C for 48 h, washed with normal saline (0.89%) three times to remove the non-adherent cells and followed by staining with 200µl of 0.1% CV in water incubated for 10min at room temperature. The plate was washed with normal saline (0.89%) and the biofilm was quantified by measure the absorbance at 550nm after solubilizing the CV by treating with 200µl of 30% acetic acid in water for 15min. Two experiments were conducted independently, in triplicate wells in each experiment [268, 269].

3.4.13 Motility Assays

Motility assay was performed to assess the effect of the silenced *csgA* gene. Isolated colonies of each strain in the study from fresh culture LB agar plates were inoculated with sterile toothpicks on swimming plates (1% bactotryptone, 0.5% NaCl, 0.5% Yeast Extract, 0.3%

Technical Agar) prepared and dried 6hr on the same day before inoculation. Plates were incubated at 37°C for 6hr. Swimming halo diameter was noted for each test. For each strains at least six colonies from each strain were tested and the test was repeated twice at least [270-272]

3.4.14 Hemagglutination Assay

Hemagglutination assay was performed in 96 well plates to analyze the expression of *csgA* gene for both knockdown and wild strains in the study. All the strains were inoculated in 5ml of brain heart infusion broth (BHI), incubated at 37°C overnight. The cultures were centrifuged at 5000rpm for 5min, supernatant was discarded and the pellet was re-suspended in Phosphate buffer saline to a concentration of 5X 10^{10} per ml (thick suspension). Micro-titer polystyrene chilled plates were taken and 25µl of chilled each bacterial strains suspension and 25 µl of 3% concentration of chilled RBCs were added to each well the, plate was incubated for 1hr at 4°C. The hemagglutination was observed as a whole even sheet of agglutinated RBCs. Wells with positive hemagglutination test were graded as +1, +2, +3 and +4, depending on upon the clumping pattern when viewed on a mirror. The results were noted for each strain of each well. For each strains at least six colonies from each strain were tested and the test was repeated twice at least [273, 274].

3.4.15 Hemolysis Assay

Hemolysis assay was performed using 96 well plates to analyze the knockdown *csgA* gene expression of the APEC strains in the study. The isolates were sub-cultured in 5ml of fresh BHI broth media overnight at 37°C with shaking. The100µl overnight culture and 1% of PBS washed human RBCs of 100µl was added to the each well in triplicate for each strains. The plate was covered by plastic Food wrap and incubated for 24hr at 37°C.if the hemolysis was not observed properly after 24hr and then the plate was incubated for another 24hr at 2-6°C for good observation of hemolysis. The control consists of fresh BHI broth and equal volume of PBS washed human RBCs. The results were noted for each well [273, 274].

3.4.16 Statistical Analysis

The significance differences in expression of *csgA* gene at mRNA level between wild type and *csgA* silenced APEC-O1 was analyzed statistically using ANOVA with a p value <0.05. Similarly, biofilm, motility and hemagglutination assays were also analyzed statistically using

ANOVA, to find out the significant difference between wild type and *csgA* silenced APEC-O1 [275].

3.4.17 Tables

Table 6: Sequences of primers for *csgA* and *uidA* genes for detection and Real Time RT-PCR

Primer Name	Sequences
csgA gene Forward	5'-GTCACGTTGACGGAGGAGTT-3'
csgA gene Reverse	5'-ATTTACCAGTACGGTGGCGG-3'
uida Forward	5'-AGACTGTAACCACGCGTCTG-3'
uida Reverse	5'-GCGAGGTACGGTAGGAGTTG-3'

Table 7: Sequences of GUIDE and TARGET sequence used for knock downing *csgA* gene

Primers name	Sequence
csgA Guide Sequence	5'-TGCTGCTACTTTTAAAAGTTTCAT-3'
csgA Target Sequence	5'-ATGAAACTTTTAAAAGTAGCAGCA-3'

Table 8: Expression cassette of sRNA included promotor, GUIDE sequence, MicC scaffold and terminator

sRNA Expression cassette	Sequence
pR-promoter	TAACACCGTGCGTGTTGACTATTTTACCTCTGGCGGTGATAATGGTTGC
Guide-sequence	TGCTGCTACTTTTAAAAGTTTCAT
MicC scaffold	TTTCTGTTGGGCCATTGCATTGCCACTGATTTTCCAACATATAAAAAGACAAGCCCGAACAGTCGTCCGGGCTTTTTTTCTCGAG
T1 Terminator	TAACACCGTGCGTGTTGACTATTTTACCTCTGGCGGTGATAATGGTTGCTGCTGCTACTTTTAAAAGTTTCATTTTCTGTTGGGCCATTGCATTGCCACTGATTTTCCAACATATAAAAAGACAAGCCCGAACAGTCGTCCGGGCTTTTTTTCTCGAG

Table 9: Sequences of primers used for Site-directed mutagenesis of expression cassette

Primers Name	Sequence
csgA Guide Forward	5'-CTGGCGGTGATAATGGTTGCTGCTGCTACTTTTAAAA GTTTCATTTTCTGTTGGGCCATTGC-3'
csgA Target Reverse	5'-GCAATGGCCCAACAGAAAATGAAACTTTTAAAAGTA GCAGCAGCAACCATTATCACCGCCAG-3'

Table 10: PCR mixture condition used for detection of *csgA* gene using primers mentioned in Table.6

Components	Quantity
Templet DNA	(500ng) 4µl
10µl primers	1µl (each)
Green Taq pol MM	12µl
Nuclease free water	7µl
Total volume	25µl

Table 11: PCR protocol for detection of *csgA* gene with a condition mentioned in Table 9

Steps	Temperature	Time
Initial denaturation	95°C	5min
30 cycles	95°C	30sec
	55°C	30 sec
	72°C	40 sec
Final Extension	72°C	5min
Storage	4°C	

Table 12: PCR mixture condition for the Site-directed mutagenesis using primers mentioned in Table 8

Components	Quantity
Templet plasmid	500ng(5µl)
10µl primers	2µl (each)
Master-Mix (MM) Phusion	24µl
Nuclease Free Water	17µl
Total volume	50µl

Table 13: PCR protocol for the Site-directed mutagenesis with the mixture conditions mentioned in Table 8

Steps	Temperature	Time
Initial denaturation	95°C	5min
30 cycles	95°C	30sec
	68°C	30sec
	72°C	2min
Final Extension	72°C	5min
Storage	4°C	

Table 14: Plasmid extraction solutions/buffers

Solution:I (Resuspension Buffer)	Solution:II (Digestion/Lysis Buffer)	Solution:III (Precipitation Buffer)
Glucose= 0.5 M	NaOH= 0.2 Normality	KAc= 5M (pH=5.6)
Tris/HCL= 0.025 M	SDS= 1% (1g/100ml)	Acetic Acid 2M
EDTA= 0.01 M		
pH= 7.4		

Table 15: Competent cell solution/buffers

Solution I	Solution 2
$CaCl_2$ = 100 mM	$CaCl_2$= 100 mM
ddH_2O	ddH_2O
Filter sterile/ autoclaved (SI and SII)	Glycerol= 15%

Table 16: Antibiotics concentration for single transformation and co-transformation of plasmids

Single Transformation	Co-transformation
Penicillin= 100µg/ml of media	Penicillin= 50µg/ml of media
Kanamycin=50µg/ml of media	Kanamycin=25µg/ml of media
	Reduce the quantity if no colonies are found

Chapter 4

4 Results

4.1 Prediction of Binding Energy for sRNA:

Binding energy of the sRNA was measured through IntaRNA tool. Repression efficiency of sRNA is related to binding energy with target mRNA. The binding energy should be in range of −20 to −30 kcal/mol, for the maximum repression [240, 276]. The free energy/binding energy was found -29.60kcal/mole. The binding site of the sRNA and target site at mRNA is depicted (fig.7).

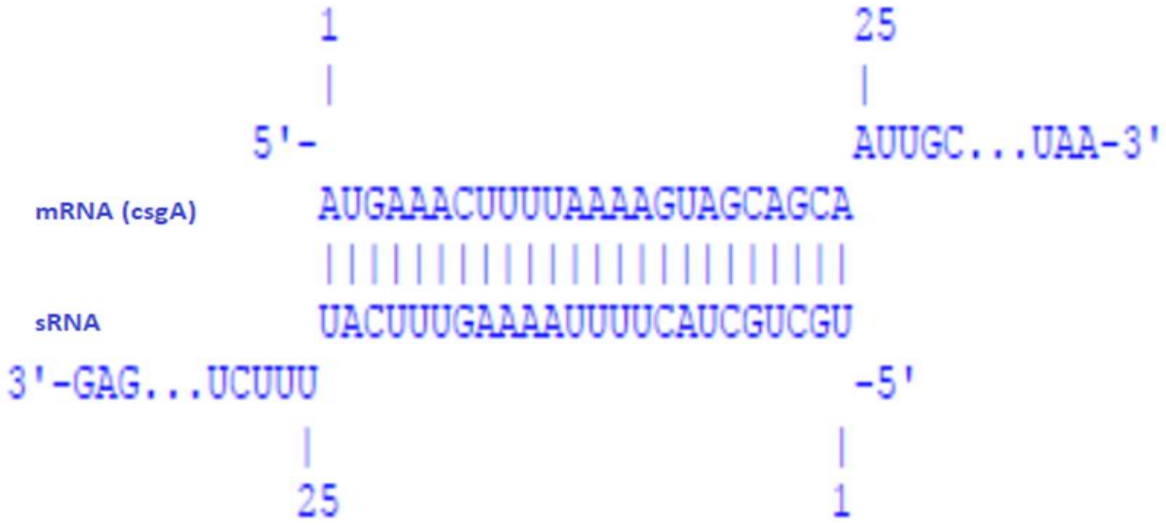

Figure 7: Predicted binding site (seed region) of the target mRNA with sRNA by IntaRNA tool.

4.2 Off Targets Prediction of sRNA

Off targets were predicted using CopraRNA. Several off targets were predicted but there binding energies were not in the limit of efficient and optimal repression of mRNAs as shown in figure 8. The off targets were based on the binding energy and none of the off targets were in the optimal binding energy range except the *csgA* gene target.

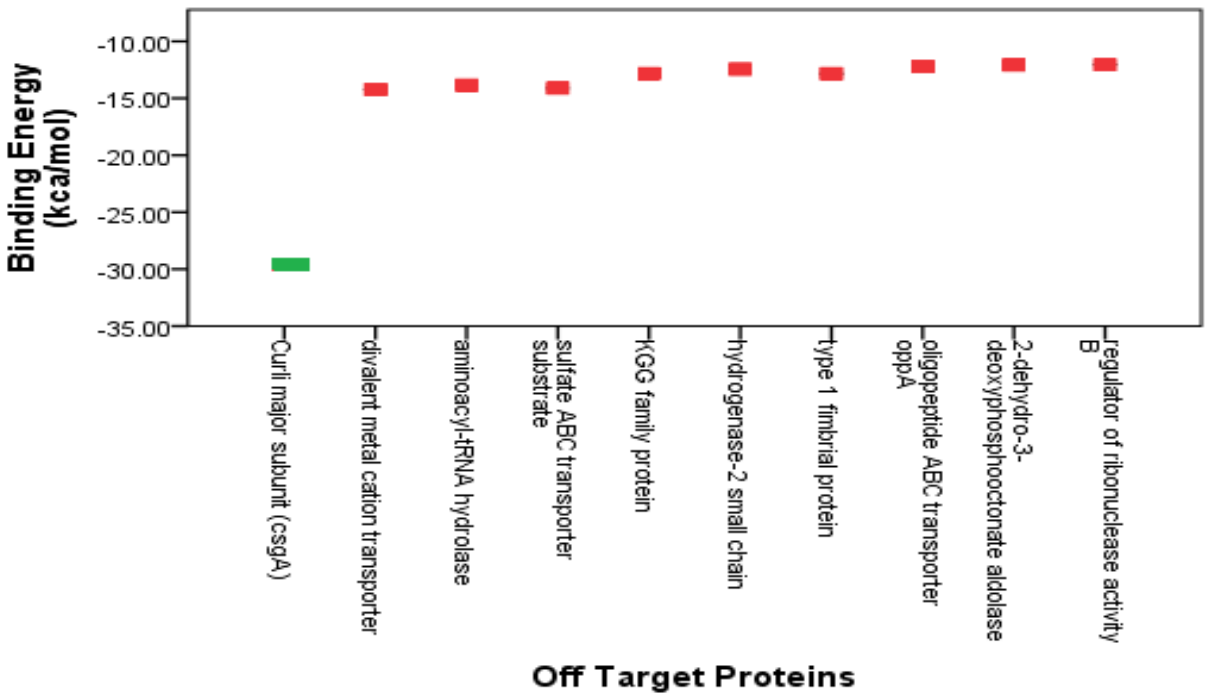

Figure 8: Off targets prediction with binding energy of the sRNA in *E. coli* strains

4.3 Secondary Structure Prediction of sRNA:

The sRNA with Hfq binding domain must have 2 to 3 stem loop structures for optimal repression of the target mRNA. The secondary structure of MicC scaffold and sRNA is essential for stability *in vivo*, facilitate Interaction with mRNA and Hfq protein recruitment [276, 277]. The secondary structure depicted by RNAfold showed three step-loops as shown in figure.9.

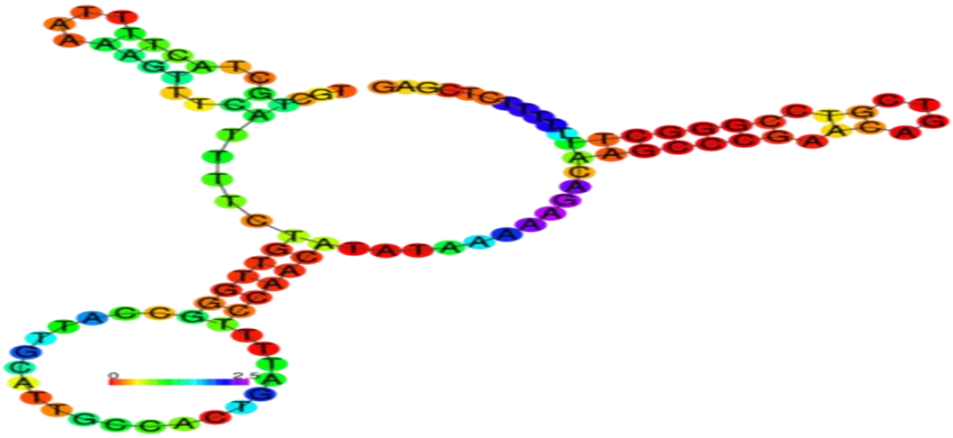

Figure 9: Secondary structure of sRNA with MicC scaffold

4.4 Detection of *csgA* gene through PCR:

DNA was extracted from the APEC-O1and run on the gel (Fig.10a). The DNA was properly extracted and the quantified DNA through nano-drop was found above 500ng. The PCR was run with suggested conditions (table) and thermos-cycler protocol (table). The gene was amplified and run on the gel (Fig.10b), the strains were found positive for *csgA* gene with amplicon size 275bp.

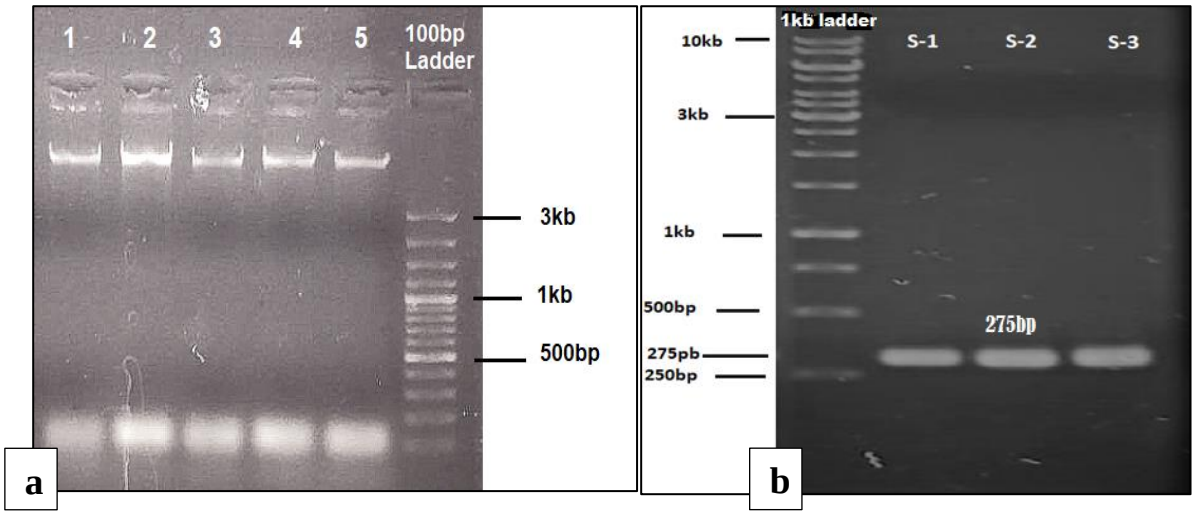

Figure 10: (a) DNA extracted from APEC-O1 (b) Amplified *csgA* gene through PCR APEC-O1

4.5 Plasmid Extraction and Confirmation:

M13KO7 helper plasmid and pAB.001 plasmids were extracted from *E. coli*DH5α, using alkaline lysis method mentioned earlier and run on the gel (Fig.11a). The M13KO7is 6.4kb and pAB.001 is 3.9kb. The plasmids size was confirmed using restriction digestion enzyme *Bam*HI and *Eco*RI respectively (fig.11b).

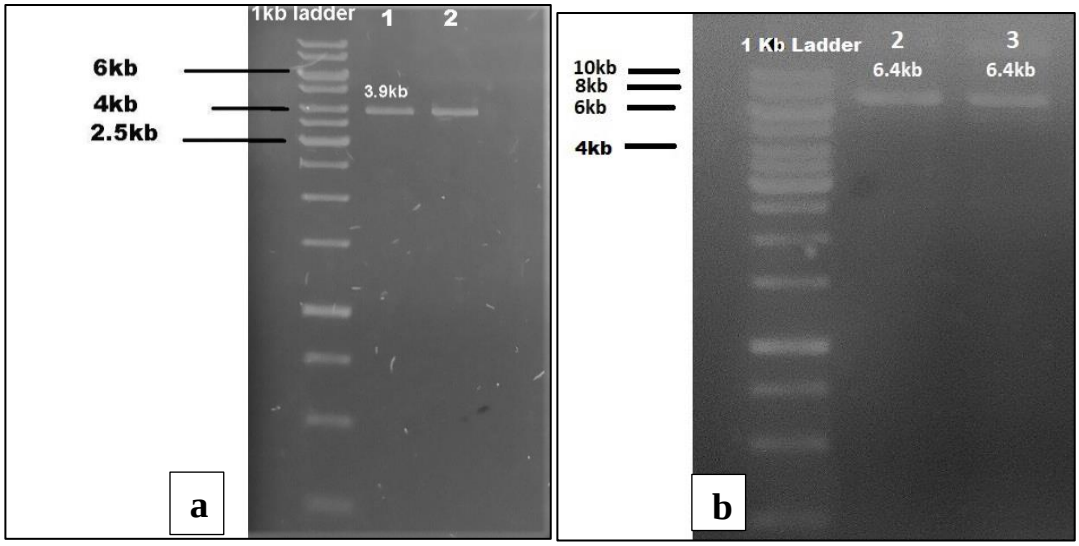

Figure 11: a) pAB.001 plasmid digested with *Eco*RI b) M13KO plasmid digested with *Bam*HI

4.6 Phagemid Modification via Site-directed Mutagenesis:

The specific primers (table.2) were used to insert GUIDE sequence into phagemid through rolling circle amplification using ordinary PCR, the suggested conditions and thermocycler protocol for site-directed mutagenic PCR are mentioned in Table.4 and Table.5 respectively. The insertion of GUIDE sequence was confirmed through sequencing by Microgen Inc, Korea. The amplified phagemid was run on the gel (see fig 12).

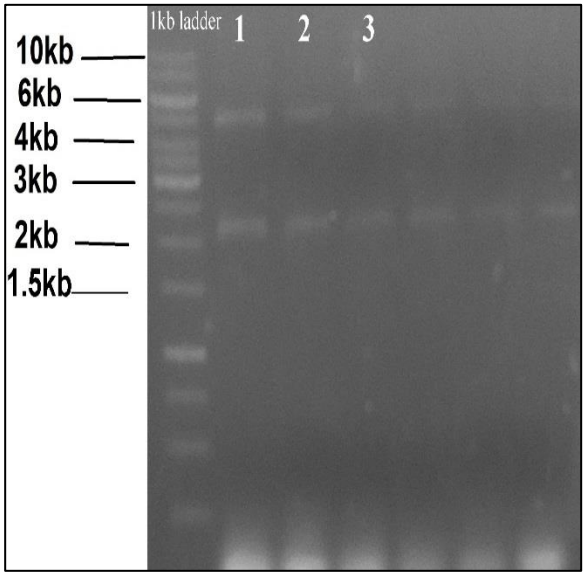

Figure 12: Site-directed mutagenesis of pAB.001 plasmid through rolling circle amplification using PCR.

4.7 Sequential Transformation of M13KO7 and Phagemid:

The plasmid M13KO7 were first transformed into *E. coli* DH5α on selective antibiotics plates, the transformed colonies were showed in figure 13a, followed by transformation of sRNA expression phagemid (pAB.001) as shown in figure 13b. The transformation of plasmids was confirmed by band size though restriction digestion**.**

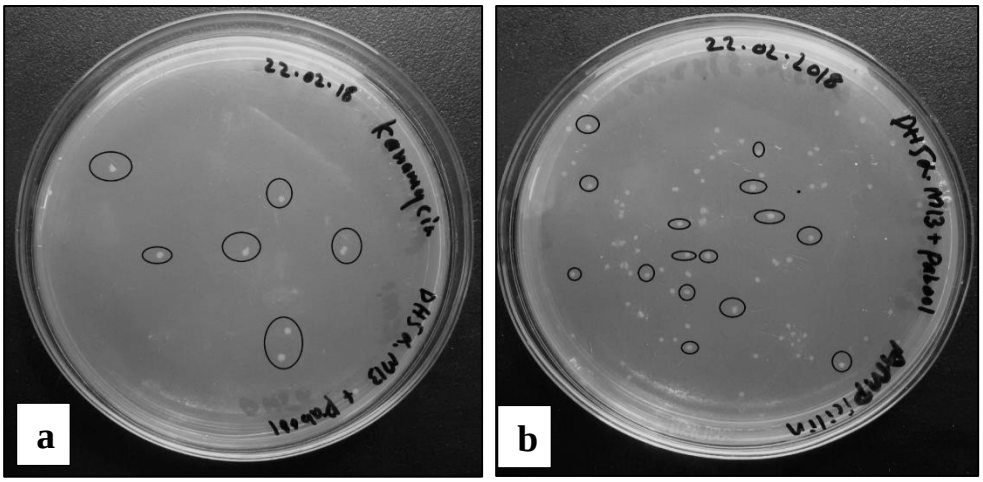

Figure 13: Transformed colonies of *E. coli* DH5α a) pAB.001 b) Co-transformed M13KO and pAB.001.

4.8 APEC-O1 Transduction with M13 Packaged Phagemids:

The M13 packaged phagemids were isolated as a filtrate and APEC-O1 cells were infected, the infected colonies were obtained (fig.14).

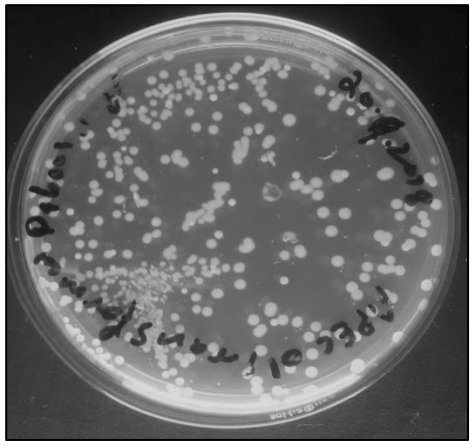

Figure 14: Colonies of APEC-O1 transfected with M13 packaged phagemids (pAb.001)

4.9 Expression analysis:

Expression of sRNA and Hfq mediated silencing of target *csgA* gene was analyzed at mRNA level through Real Time RT-PCR. In Addition, the effect of *csgA* gene was further investigated for biofilm formation, motility assays, hemagglutination and hemolysis assay.

4.9.1 Expression analysis of *csgA* gene via Real Time RT-PCR:

Results showed that a significant reduction was observed of *csgA* gene in phagemid infected APEC-O1 as compare to wild type APEC-O1. The data was normalized with housekeeping gene *uida*. The fold change was measure through Livak method and almost 5-fold change was observed which indicate about 43% reduction of *csgA* gene at mRNA level. The relative expression ratio of wild type observed 1 and *csgA* silenced APEC-O1 was 0.179, depicted in figure 15.

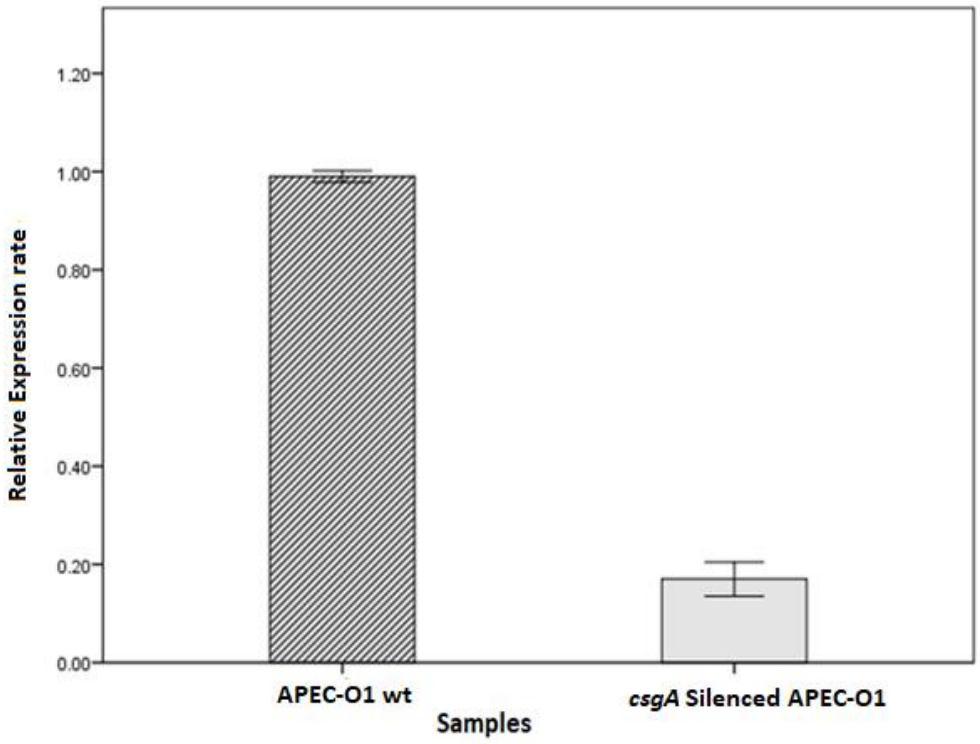

Figure 15: Relative expression rate of APEC-O1 wild type compare to *csgA* silenced APEC-O1

4.9.2 Biofilm analysis of wild type and *csgA* silenced APEC-O1.

Biofilm assay was performed to know the effect of curli fimbriae in biofilm formation and results showed a significant reduction of biofilm was observed in phagemid infected APEC-O1 (p=0.0001), as compare to wild type APEC-O1, as depicted in figure 16.

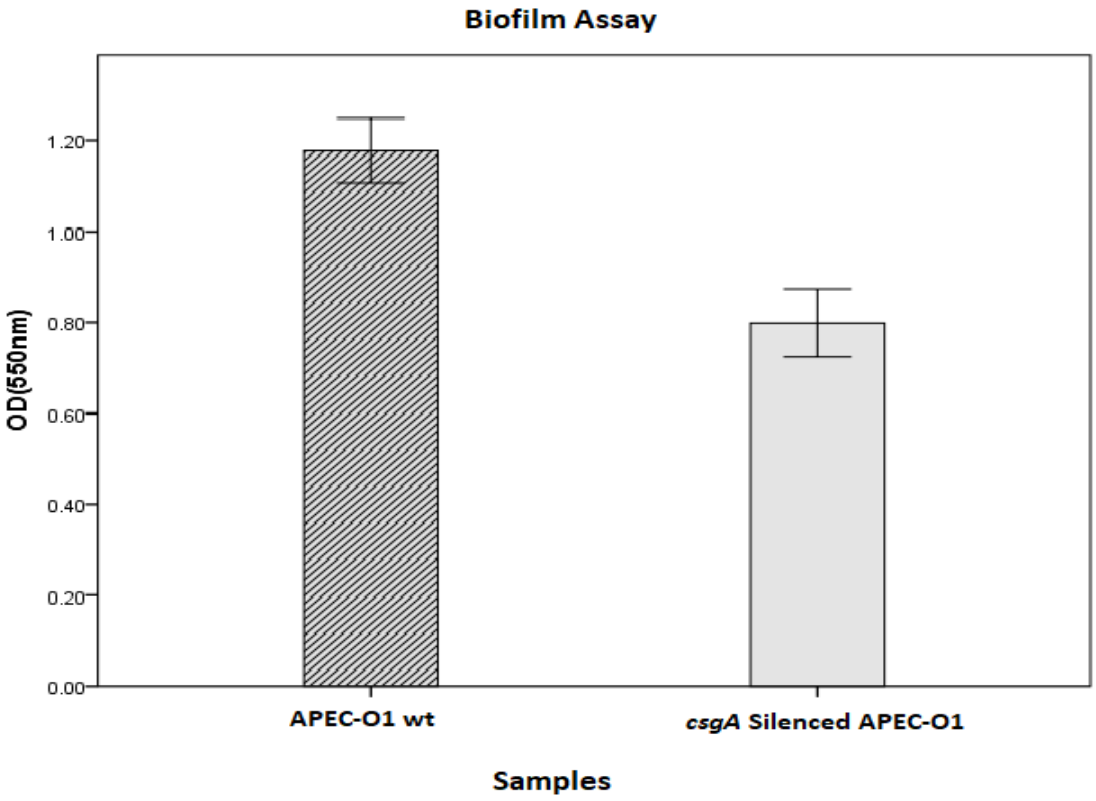

Figure 16: Analysis of the biofilm formation by APEC-O1 wild type and *csgA* silenced APEC-O1

4.9.3 Motility analysis of wild type and *csgA* silenced APEC-O1:

Swarming motility is a flagellar movement, restricted by pili and fimbriae. Silencing of curli fimbriae may increase the swarming motility of bacteria. Results showed that the motility was significantly increased in *csgA* silenced APEC-O1 as compare to APEC-O1 wild type ($p<0.05$). The motility of *csgA* silenced APEC-O1 was about 6mm while the wild type was measured 3mm (see fig. 17).

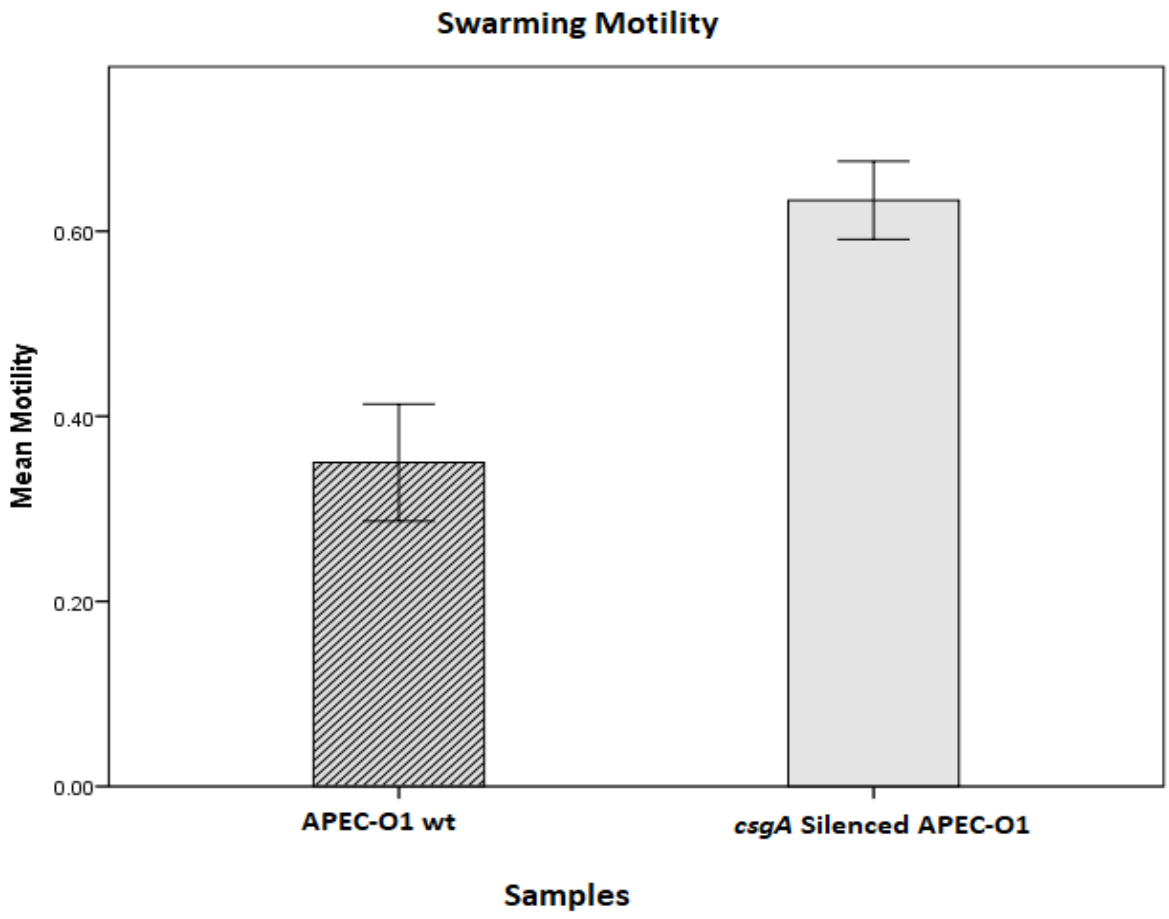

Figure 17: Swarming motility of wild type and *csgA* silenced APEC-O1.

4.9.4 Hemagglutination of wild type and *csgA* silenced APEC-O1:

Hemagglutination assay was performed to observe the adherence of wild type and *csgA* silenced APEC-O1 to erythrocytes. The results showed that the adherence differences were not significant in between wild type and *csgA* silenced APEC-O1 (p=0.182). The Hemagglutination values were +3.3 and +3.2 for wild type and *csgA* silenced APEC-O1 respectively, depicted in figure 18.

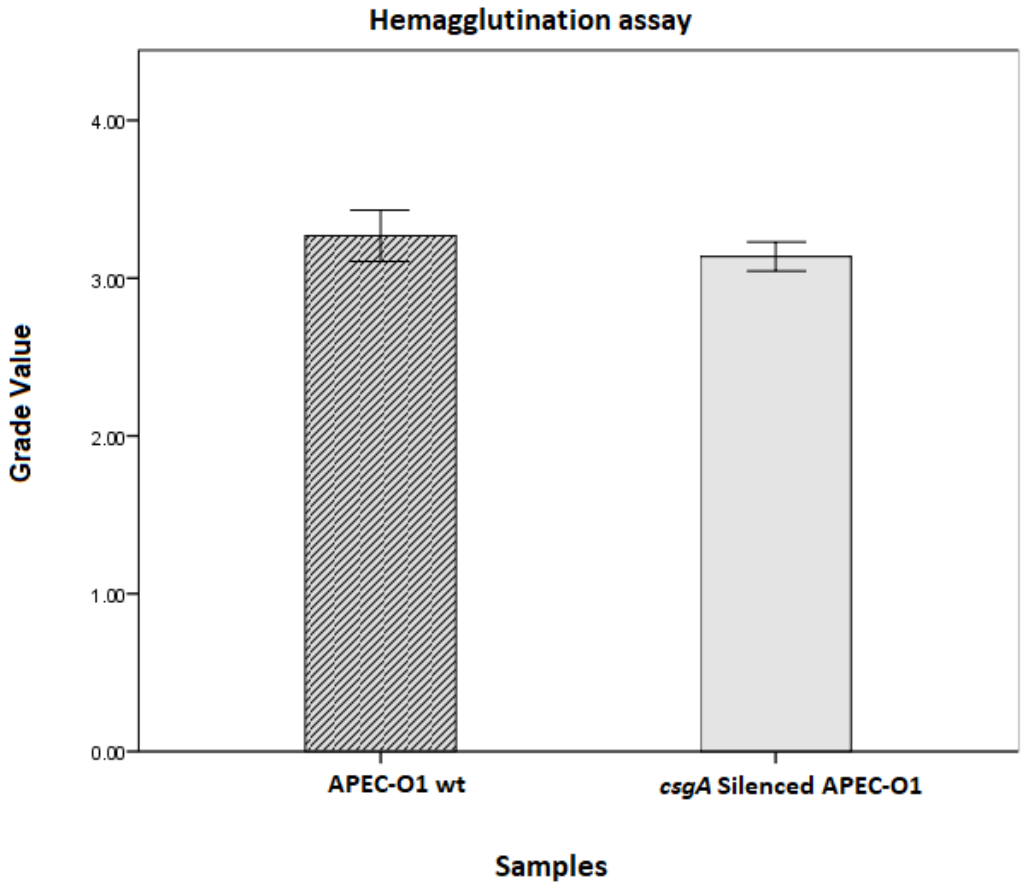

Figure 18: Hemagglutination assay of wild type and *csgA* silenced APEC-O1

4.9.5 Hemolysis of wild type and *csgA* silenced APEC-O1

The virulence of *csgA* was investigated through hemolysis assay. The results showed no significant difference in hemolysis of wild type and *csgA* silenced APEC-O1. The hemolysis type was subtle, depicted in figure 19.

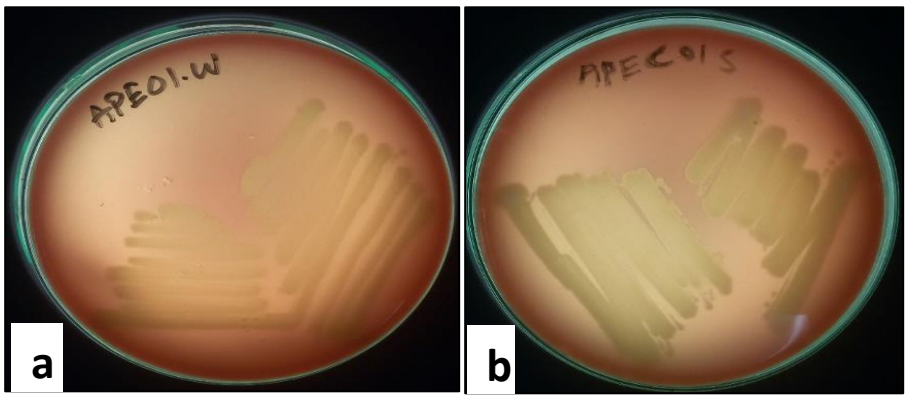

Figure 19: Hemolysis assay a) APEC-O1 wild type b) *csgA* silenced APEC-O1

Chapter 5

5 Discussion

Treatment of poultry infections through antibiotics and vaccines is facing problems due to the bacterial resistance and variants of pathogenic bacteria. Novel antibacterial strategies are nowadays evolving and at developing stage, an efficient and appropriate treatment for bacterial infections. Beside phage therapy, this study suggests that M13 phagemid mediated therapy, has the potential to treat bacterial infections. M13 phagemid mediated therapy, comprised of Hfq mediated sRNA expression cassette which is incorporated in a phagemid, an expression vector. the expression vector is delivered by the M13 helper phage into the target host [20]. The study included, designing of sRNA for the target gene (*csgA*) and their computational analysis to predict the binding energy, secondary structure and off targets with in the host. Beside *in silico* analysis, the study includes sRNA expression cassette modification of the phagemid and phagemid delivery through M13 helper phage into the target host to knockdown the *csgA* gene *in vitro.* Real-time RT-PCR further analyzed the target gene repression and the gene repression was tested for biofilm formation, motility, hemolysis and hemagglutination.

The M13 helper-phage production leads to mutation at high rate and plasmid loss due to metabolic burden. It was observed in our study as reported in a previous study that frozen and refrigerated strain has low number of M13 packaged phagemid production and less efficiency of infection, [20]. The co-transformation was a critical step; a very low efficiency has been observed transforming both plasmids (pAB.001 and M13KO) simultaneously as previously reported [20, 244]. The high rate of transformation of both plasmids was achieved by sequential transformation, the M13KO plasmid was transformed first, then making the cells competent again and followed by phagemid (pAB.001) transformation. Infection with M13 packaged phagemid has also changes metabolic pattern of the host while targeting specific gene by tittering Hfq protein at high level and may lead to off target silencing can also change gene expression profile. It is reported in a previous study that bacteria was found resistance to M13 phage by mutational loss of pili, which may reduce the potential of the given strategy [243]. The chance of occasional transfer of M13KO7 plasmid may compete the

transfer of the phagemid into the M13 capsid at a low rate, may lead to spread of phages with continuous level with in the target. In addition, the infection efficiency was less than 100 percent, which allowed some bacteria to persist within the infection target.

The Hfq mediated sRNA through M13 phage delivery to modulate gene regulation in *E. coli,* is simple, fast and adoptable to wide range of targeting genes. The target host (*E. coli*) is not burdened elsewhere phagemid proliferation and sRNA expression. Phagemids can be easily modified through single PCR to target nearly any gene, and can be produced before phagemid transformation, a day after. The sRNA expression cassette was found with greater than 90% of typical repression level, work on different rang of metabolic targets, on a standard plasmid vector, which is somehow correlate with the present study [240]. The same strategy was applied to silence the target gene in a previous study; the repression was observed about 80% with mKate fluoresce gene as measuring standard while in this study the repression was about 45% using Real-time RT-PCR. The decrease in repression efficiency was to be the cause of nonspecific off targets binding and sRNA mutation while introduction into phagemid using rolling circle PCR [20].

The target gene *csgA* has been studied previously and it was observed that the gene has major role in biofilm formation, adhesion, motility and colonization. The present study correlated with the previous study in which the *csgA* gene knockout in O45:K1:H7 strain of UPEC (Uropathogenic *E. coli*) which didn't express the curli fimbriae failed to Congo red dye binding has shown higher biofilm formation in salt deficient LB condition, which induces curli fimbriae production [278]. The same study also observed reduction in colonization in *csgA* mutant strain in bladder infection, the data combine with biofilm formation showed the involvement of *csgA* gene in virulence. Another study showed that *csgA* mutant of UPEC-4 strain did not continued to adhere with HTB-9 cell line (human bladder carcinoma) and reduced adhesion to Vero cell line. The present stud showed that the adhesion with RBCs through hemagglutination assay was not significantly reduced. The motility of Pseudomonas spp. was investigated in a previous study and results showed that type VI pili mutant tend to spread more rapidly, while the wild type tend to swarm or tendrils [279]. While in our study, the curli fimbriae silenced APEC strain showed more spreading then wild type APEC but still

we need more investigation and research to confirm the relation of curli fimbriae with motility and colonization.

The methodology used in this study has the potential to reduce the virulence at maximum level by targeting virulence gene, responsible for infection and pathogenicity. Previous study has successfully silenced the antibiotic resistant genes, which make the target bacteria susceptible to the antibiotic. The main limitation of this strategy is that this strategy is limited to those species that have Hfq mediated silencing system. Secondly, the feedback mechanisms overcome the repression of a single gene. It is necessary to target those genes that can reduce virulence and pathogenesis at a significant level.

Chapter 6

6 Conclusion

It is concluded form the study that, the designed strategy has the potential to silence the target gene at a significant level. The design sRNA repressed *csgA* virulence gene up to 45% at mRNA level. Further the *csgA* silenced APEC-O1 reduced the biofilm of about 34% compare to wild type APEC. The motility and adhesion was also affected at certain level. As the reduction in biofilm formation and adhesion is concern with pathogenies, the current study suggests that targeting curli fimbriae may reduce the virulence and pathogenicity of APEC. It is also concluded that the limitations of the strategy reduced the efficiency and applicability to broad host range and silencing of the target gene. However, phagemid therapy has the potential to treat infection and combat against antibiotics resistant *E. coli*.

Chapter 7

7 Future Prospects

Future prospects include further investigation of the curli fimbriae effect on virulence and pathogenesis and phages infection efficiency *in vivo.*

- Curli fimbriae silenced APEC should be further tested for pathogenicity on specified cell lines and *in vivo* (chicken).
- Efficiency testing of M13 packaged phagemid infections carry out *in vivo* to evaluate the infection rate and route of phages infection.
- By targeting other virulence genes of APEC to reduce virulence and pathogenicity at higher level.
- It is also possible that phagemid would transfer the gene into chromosome of the target host so it is necessary to test for phagemid antibiotic gene transfer to host genome.

Chapter 8

8 References

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