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PRODUCTION OF RECOMBINANT PROTEIN 'FLIGLO' FOR THE DETECTION OF *ESCHERICHIA COLI* O157:H7

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ABSTRACT: *Escherichia coli* O157:H7 is a very common food borne pathogen. Without the detection at the right time, it could turn fatal. It is not only diagnosis, but the trials to make it non-virulent are also important. Detection of this in humans is critical, since appropriate therapy can be administered. Here we demonstrate a system appropriate for *E. coli* O157:H7 that can be extended to other bacteria and viruses such as HIV, MRSA detection. The gene *fliH, in E. coli* O157:H7, which is one of the major genes that produces the protein necessary for the association of the flagella, was targeted in this study. A recombinant protein (for *fliH*) that tagged with GFP protein was produced using the plasmid. Green fluorescent protein (GFP) obtained from GFP gene is known for its fluorescence. This makes it very useful in molecular biology to detect the target gene when it is tagged to GFP. The *fliH* gene, which plays an important role in flagellar association in the bacteria was used here. This when tagged with GFP is an important diagnostic tool for detecting the presence of *E. coli* O157:H7 in the samples. For preparing this system, *E. coli* O157:H7 DNA was isolated and restriction digested and by using other techniques, the *fliH* gene was introduced into the pGLO plasmid. This plasmid was expressed to produce the fusion protein 'fliglo'. The isolated fliglo fusion protein has different spectral signature than the original GFP. GFP shows an emission peak at 509 nm, whereas the fusion protein shows it at 461 nm. This is useful in isolation of pure fusion protein. Primer designing was done using the bioinformatical tools, followed by sequencing. The results showed that there was successful insertion '*fliglo*' gene to produce fliglo protein. Further the fusion protein system will be utilized for other bacteria and virus detection in blood samples and also the production of highly specific antibodies for medical applications.

KEYWORDS: *Escherichia coli* O157:H7, *fliH,* GFP, pGLO, restriction digestion, fliglo protein, primer designing and sequencing.

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1.INTRODUCTION

Some strains of *Escherichia coli* O157:H7 produce peptide enterotoxins that cause bloody diarrhoea in humans. They can become a major cause of infectious diarrhoea throughout the world, and they represent a serious health hazard to humans. Along with bloody diarrhoea, cases which leads to kidney failure, especially in young children and elderly people have also been reported[1]. The illness caused by this strain can often be life threatening. In such cases just treatment will not be sufficient. Finding out new techniques of fighting the bacteria also becomes important. *Escherichia coli* O157:H7 grow in the colon and the symptoms mainly include fever, chills, headache, abdominal cramps, diarrhoea, dehydration and vomiting. In spite of various efforts to control the disease, *Escherichia coli* has remained a major threat worldwide. In India, *Escherichia coli* O157:H7 has recently been isolated from dairy cattle and beef in Kolkata[2,3]. Besides the conventional pathogens which are transmitted by water, several emerging water-borne pathogens have become increasingly important during the last decade in India. These include Shiga toxin producing *Escherichia coli* especially Enterohaemorrhagic *Escherichia coli* (EHEC). This test was done in Delhi, test being conducted on the upstream Yamuna river water[3]. EHEC O157 has been isolated from the Ganges River, Varanasi[2]. In Mangalore, Karnataka, a study done in Kasturba Medical College showed that out of the 192 stool samples collected at random, 120 samples showed positive for *Escherichia coli* O157:H7. Even the meat samples were also checked which were picked from stores in various areas. Here among the 103 meat samples, 65 were positive for *Escherichia coli* O157:H7[4]. Since the emergence of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) serotype O157:H7 as an agent of hemorrhagic colitis and the hemolytic-uremic syndrome (HUS) in 1982, hundreds of outbreaks of disease associated with enterohemorrhagic *Escherichia coli* (EHEC) have been reported in the United States, including a large multistate outbreak associated with bagged spinach[5]. Isolation rates have been widely reported, with levels of 1.8% in Japan, 1.9% in Australia and 1% in Mexico. Food is not the only source for the spread of *Escherichia coli* O157 strain. There is another major source and that is water, especially the water in the swimming pool. In June 1993, 4 children, aged 1.5-3.5 years admitted to a hospital in Netherlands were diagnosed with hemolytic uremic syndrome (HUS) within one week. The reason behind this was that all these four children had bathed in the same, shallow, recreational lake within a period of 5 days[6]. The development of vaccines against the most common etiologic agents of Gram-negative sepsis (e.g., *Escherichia coli*) or of antibodies for passive

immunization is therefore an important objective, despite the availability of potent antibiotics[7]. Polyclonal antibodies are not specific, due to the use of whole cell antigen. Monoclonal antibodies can be highly specific and its production is more essential to treat the symptoms caused by *Escherichia coli* O157:H7. Mostly they are produced in the lab animals like mice if it is small scale. But there is a need of production of antibodies in a large scale for use in diagnostics, which makes use of IgG from serum. But there are certain drawbacks in producing the antibodies in mammals. Firstly, bleeding is considered painful for the animals and hence is not recommended for the welfare of the animals[8]. Secondly, the amount of IgG obtained from serum is very less. It is found that antibody produced from one rabbit is as less as 16.6mg. Hence it is not found to be economical[9]. In such cases chicken can be used as an alternative for large scale production. Chicken egg yolk (IgY) has the advantage over mice of not having phylogenetic similarity with the mammals when tested on the humans[9]. It is also found to be economical than producing in mammals. However egg yolk is a fluid emulsion with a continuous phase of protein and a dispersed phase of lipoprotein particles[10]. Hence the biggest obstacle for isolation of chicken antibodies is the removal of lipids, which are present in abundance in egg yolk, which can be a major drawback in IgY production[8]. To overcome all the above mentioned problems, production of recombinant protein is the next alternative. Production of recombinant proteins requires isolation of DNA as an initial step. It is however necessary that all the contaminants (proteins, lipids and RNA) should be removed before this process begins. Once this is done, restriction digestion is performed by using a specific set of enzymes called restriction endonucleases. Restriction endonucleases recognize specific 4-base (tetramer), 5-base (pentamer), or 6-base (hexamer) sites located on the incoming DNA, and make double-stranded cuts. The nature of restriction endonucleases is that they are palindromic (reading same from both ends). EcoRI is one of the restriction enzymes that is extensively used. In the EcoRI, the cuts are made to the left of the axis of rotation, producing 5' overhangs. A plasmid (vector) is required to insert the gene of interest (insert). The isolated gene will be inserted into the cut ends of the plasmid. This will be followed by ligation which is done by another enzyme, ligase. Ligation is a process where the linear plasmid vector and recombinant (vector-insert) DNA molecules are formed during *in vitro* which in turn will lead to formation of circular recombinant molecules[11]. Now this sealed plasmid needs to be introduced into the bacteria in a process called transformation. In one of the experiments E.coli JM109 cells were used for transformation. pUC19 plasmid was used to insert the gene of interest. The efficient introduction of DNA into bacteria are performed by methods like treatment with $CaCl_2$ and electroporation[12]. In order to make sure that the recombinant plasmid has been taken up by the bacteria, it is also important that the gene must be tagged with either a radio isotope or a fluorescent material. The latter is always easier as most of the time the result can be seen by the naked eyes. One of the best examples for this is use of *gfp* gene of plasmid pGLO. *gfp* gene was originally

isolated from the jellyfish, *Aequorea victoria*[13]. Specific mutations were introduced into the DNA sequence, which greatly enhance fluorescence. This becomes an easy tool to know the result of transformation. One of the studies which included random DNA fragmentation with endonuclease V showed use of *gfp* gene[14]. Once a successful insertion is confirmed, extraction of protein is done. Hydophobic Interaction Chromatography (HIC) is one of the many protein purification methods. This either is done at high salt concentrations or high temperature to strengthen the hydrophobic interaction. In one of the studies, *Bacillus horikoshii* was used to produce many enzymes like proteases and lipases. It made use of HIC for the purification of those enzymes[15]. The present study focuses on *fliH*, an important flagellar assembly gene of *Escherichia coli* O157:H7 being inserted into the pGLO plasmid next to the *gfp* gene to produce recombinant gene, which was called '*fliglo*' gene. This recombinant gene was transformed to produce 'fliglo' protein. The optical density of the fliglo protein was measured in a fluorometer and the emission peak of the 'fliglo' protein was measured. Through this we demonstrate a method that can not only be extended to other pathogens but also that will help in a timely prognosis and treatment of various harmful diseases caused by bacteria and viruses.

2. MATERIAL AND METHODS

Isolation of genomic DNA of *Escherichia coli* O157:H7 (ATCC 43895) was done by using DNA purification Wizard genomic kit (PROMEGA kit cat no. A1120) and plasmid pGLO was isolated using Plasmid DNA Purification QIAprep kit (QIAGEN cat. no. 27104). Once the DNA is isolated, the Nanodrop (THERMO SCIENTIFIC cat no. ND2000) was used to measure the concentration of the DNA. 5µl of isolated genomic DNA and plasmid DNA were run on separate 1% agarose gel with 1mg/ml ethidium bromide. Restriction enzyme EcoRI-HF (NEB cat.no. R3101T) was used for restriction digestion of both *Escherichia coli* O157:H7 DNA and plasmid DNA. Enzyme concentration range chosen for digestion of *Escherichia coli* O157:H7 DNA was 0.5 - 4U and the time range was 0.5 - 2.5hr. This was to obtain the fliH gene. Enzyme concentration range chosen for digestion of plasmid DNA was 0.5-2U while the time range was 15 min, 30 min and 1 hr. To confirm the success of restriction digestion, denaturation gel was used. This was applicable for all the concentrations of genomic DNA and plasmid DNA. The isolated *fliH* (insert) gene was inserted into the pGLO plasmid (vector) next to the *gfp* gene. The nicked region was ligated. Ligation was carried out according to the protocol given in T4 DNA ligase (NEB M0202). However there were two changes done here. The molar ratio of the vector (pGLO) to insert (fliH) was maintained as 1:1, instead of 1:3 as mentioned in the protocol. The total reaction mixture was maintained at 50µl unlike the prescribed protocol of 100µl. The ligated pGLO plasmid DNA, which now has *fliH* gene in it was extracted from the gel according to the protocol given in the Gel extraction kit (AXYGEN cat. no. AP-GX-50). The ligated pGLO plasmid with pGLO containing the *fliH* gene was transformed into the overnight grown culture of non-virulent strain of *Escherichia coli* (ATCC 25992). A loopful of

plasmid containing the *fliH* gene was placed in the 4ml of TSB media and incubated at 37ºC for overnight incubation. A loopful of the same mixture was spread on TSA plate and incubated at 37ºC overnight. 100µl of 10mM lysozyme was added to the overnight grown culture in the TSB media. The cells were lysed using the freeze – thaw method. This was followed by separating the soluble fraction from the insoluble cell lysate is centrifugation. The supernatant obtained will contain the recombinant protein, which from now on will be referred to as 'fliglo' protein. This supernatant was purified on a Hydrophobic Interaction Column (HIC) according to the protocol of GFP purification kit (BIORAD cat no. 166-0413) to separate the fliglo protein. Along with the transformed cells, procedure from lysing of the cells till the separation of the protein on the HIC column for the non-transformed bacteria *Escherichia coli* 25992 and plasmid pGLO were performed simultaneously. The final optical density (OD) readings of all the proteins were checked on the fluorometer. Once all the results were taken, a graph was plotted with the wavelength on the X- axis and the intensity of the fluorescence on the Y- axis. For further confirmation, fliglo gene was sequenced (data not mentioned).

3. RESULTS AND DISCUSSION

DNA of *Escherichia coli* O157:H7 was isolated using the Promega Kit for isolation of the genomic DNA. The isolated DNA was checked for its concentration on the Nanodrop. Isolated DNA is prone to degradation, hence it was stored in at 2-8ºC if for immediate use. Otherwise it was used at -20ºC. Plasmid pGLO DNA was isolated as described in the kit protocol. The concentration of the DNA was measured and then stored at -20ºC. To know the quality of the isolated DNA of both genomic and plasmid, 5µl was run on an agarose gel which had 1mg/ml ethidium bromide. Clear bands were seen when observed under the UV light. This indicated that the quality of the DNA was very good. In the restriction digestion, by EcoRI-HF, among the enzyme concentrations range 0.5-4U enzyme and with the time range chosen was 0.5-2.5hr, it was observed that 1U enzyme concentration gave best digestion results for *Escherichia coli* O157:H7 DNA when digested for 1hr (Fig 1). Similarly it was concluded that for plasmid pGLO DNA, among the enzyme concentrations range 0.5-2U enzyme and with the time range chosen being 15min, 30min and 1hr, 1U of enzyme concentration for 30min gave best results (Fig 2). The confirmation was done by denaturation gel, which gave positive result that the restriction digestion was successful for both *Escherichia coli* O157:H7 DNA and plasmid pGLO DNA (Fig 3,4).Once digested, the fragment obtained from genomic DNA (*fliH*) was ligated into pGLO plasmid DNA where the nick from the restriction enzyme EcoRI-HF was made. This recombinant gene was given the name '*fliglo*'(Fig 5). T4 DNA ligase was used for the ligation. The changes that were made in ligation protocol proved to be successful than the original protocol. 1:1 ratio of vector and insert showed better results than 1:3 ratio. Total reaction mixture of 50µl worked better than 100µl reaction mixture and showed better bands. The recombinant gene *fliglo* was extracted from the gel. The gene band was compared with the ladder which was found at the expected

and calculated position (Fig 6). There was a successful ligation of *fliH* into the vector pGLO. This was followed by transformation of *fliglo* fragment into the *Escherichia coli* 25992 was performed. *Escherichia coli* 25992 was grown overnight. The following day after the inoculation of the recombinant plasmid with *fliglo*, the culture showed the light green colour glow. This green glow was not as prominent as that of pGLO plasmid, however as indicated the light green glow was clearly seen. The isolation of the protein was done as described. It showed a positive reaction which is very evidently seen in the graph plotted when the OD was measured on fluorometer. It proved the results seen in the overnight grown culture of both fliglo and pGLO. There was one more prominent observation that was made in the OD readings. Fluorometer reading showed that fliglo showed a shift in the wavelength (OD) to 461nm from 509nm. 509nm is that wavelength which is very specific for pGLO plasmid (Fig 7).

DISCUSSION

In Escherichia coli, all of the external components are translocated into a central channel by an export apparatus that is within an annular pore in the basal body of the flagellum. FliI is a flagellum-specific ATPase and FliH is a regulatory protein which prevents FliI from hydrolyzing ATP until the energy can be used for export. A tentative model for the flagellar export process has been proposed in which a (FliH)2/FliI heterotrimeric complex is present[16]. It has been shown FliH–FliI complex is important for this translocation of the external components[17,18]. Due to removal of fliH gene from Escherichia coli O157:H7, FliH protein is not formed and there is no formation of the heterodimer. This brings down the rate of swarming as well as the nutrient uptake[19]. DNA of both Escherischia coli O157:H7 and plasmid pGLO were isolated. The isolated DNA was checked for its concentration on the Nanodrop. Nanodrop method has the capacity of detecting the DNA even in a very minute volume of 1µl. The detection of DNA up to this low concentration in spectrophotometer is normally not possible. Restriction digestion is an enzymatic technique which can be used for cleaving DNA molecules at specific sites[20]. The cleavage method makes use of an important class of DNA-cleaving enzymes isolated primarily from various bacteria. These enzymes are called restriction endonucleases or restriction enzymes, and they are capable of cleaving DNA molecules at a particular short sequence of bases. Restriction digestion was experimented on both genomic DNA and plasmid DNA to get positive result on both of them. EcoRI-HF was the restriction enzyme used here. EcoRI-HF is a sticky end restriction enzyme where the cut places each strand of the DNA symmetrically around the centre of symmetry[20,21]. When any enzyme is considered for the experiments, enzyme kinetics becomes important. Like the study of the growth of the microorganism in growth kinetics, studying various parameters of the enzyme becomes very important to know the right time and the right concentration of restriction enzyme that can be used for further processes. After all the trials on different timings and concentrations of enzyme, the final result was obtained. The result

indicated that the digestion with 1U enzyme concentration for one hour was sufficient for genomic DNA and 1U of enzyme for 30 min for pGLO plasmid DNA. These concentrations and timings were used for all further purposes. fliH gene (insert) was inserted at the nicked position next to gfp gene (vector) of plasmid pGLO. This was followed by sealing the nick. Ligase is the enzyme that will seal the DNA after base pairing. This is called ligation. There are chances that the tested protocols does not become applicable to all the experiment. In this experiment, to suit the protocol being used for ligation, two changes were made. The vector – insert ratio was changed to 1:1 instead of prescribed 1:3. It was shown that the result seen after the change was better. One more change made was reduction of the reaction mixture to 50µl from prescribed 100µl, which proved to be successful. This plasmid contains the gene of interest fliH. This plasmid is now be called recombinant plasmid DNA. Plasmid cannot grow on its own. It requires host bacterial cells. Hence the recombinant plasmid was introduced into the host cell. Non-virulent Escherichia coli (ATCC 25992) was chosen as host cell. Introducing recombinant plasmid into the bacterial cell is called transformation. Transformation is the genetic alteration of a cell[22]. It includes the incorporation and expression of exogenous DNA. While it occurs naturally in bacteria, it can also be artifically induced. However a gene from a bioluminescent jellyfish and its Green Fluorescent Protein (GFP) comes as a solution to this problem. The gene for GFP was originally isolated from the jellyfish, Aequorea victoria[13]. Specific mutations were introduced into the DNA sequence, which greatly enhance fluorescence of the protein. This becomes an easy tool to know the result of transformation. Several chemicals have been used for inducing the bacterial cell transformation. But in one of the classic experiments, Mandel and Higa demonstrated that treatment of Escherichia coli with CaCl2 made the cells susceptible to uptake bacteriophage DNA[11]. Tryptic Soy Agar (TSA) plates were used for growing the bacteria, now with the recombinant plasmid. Following day, the plates when checked for fluorescence gave a light green glow in the colonies, although not as prominent as the normal GFP protein glow. This protein produced here was named as ‘fliglo’, fli from fliH and glo from pGLO plasmid. However more tests were required to prove that the transformation was successful. The separation of fliglo protein is the best way to know this. For the purification of the fliglo protein Hydrophobic Interaction Chromatography (HIC) was used. The protein was lysed and the soluble fraction was separated from the insoluble fraction as described in the protocol. After every step the reading of the concentration of protein was taken on the fluorometer and also on the Nanodrop. The Nanodrop will give the reading of the proteins in ng/ml. Although the reading is obtained, the actual way of getting the detailed and reliable result is on a fluorometer. Fluorometer is an instrument that senses the fluorescence and since the gene under consideration is having the GFP, the fluorescence will be detected. It is a better and more reliable way of knowing the exact OD of the protein. It also gives an idea of the correctness of the experiment. While pGLO showed a very high value at 509nm bacterial value was very low.

The fliglo value (461nm) was in between these two, however more towards the bacterial value. These values mainly indicated that the ligation was successful. It is the third tube which contained the protein (eluate) that will be run on the HIC column to detect the fluorescence. Green colour of the solution in the third tube indicated the success of the reaction. The solution in the third tube showed the highest value of protein, which again indicated the success of the reaction. Sequencing is one of the major methods that can confirm the successful insertion of the gene fliH into the plasmid. It will also confirm the production of the gene fliglo. Hence the fliglo was sequenced which also gave positive results. FliH can be useful in diagnostics as its overexpression can bring about detection of pathogens in both food and water samples [16, 17].

CONFLICT OF INTERESTS

The authors declared that they have no conflict of interests.

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Figures

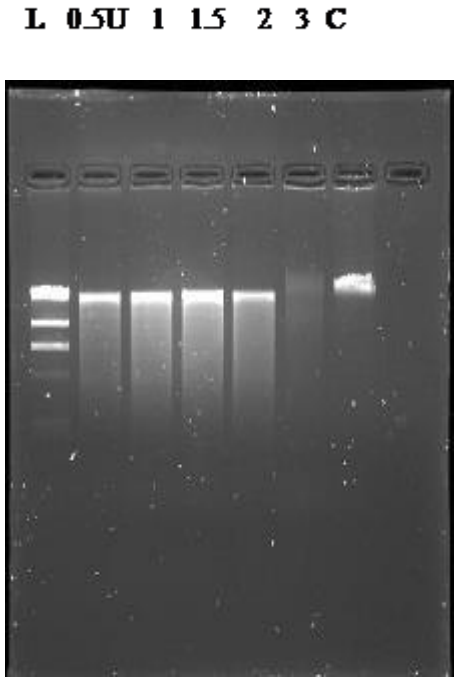

Fig 1 - Restriction digestion of E.coli O157:H7 DNA by EcoRI-HF – enzyme concentration 0.5U – 3U (L-ladder, C-control)

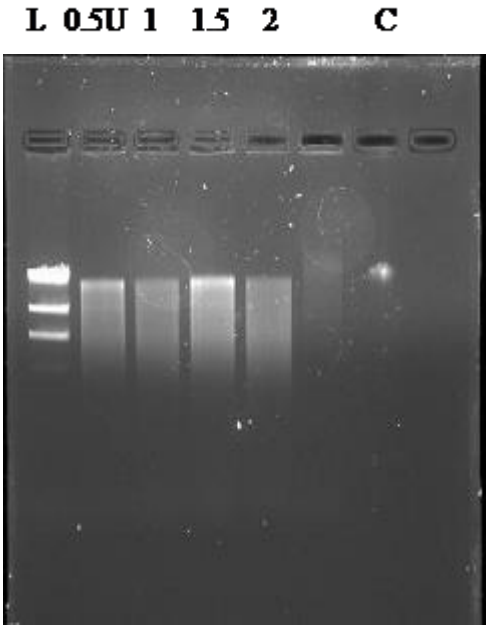

Fig 2 - Restriction digestion of pGLO plasmid DNA by EcoRI-HF tried for 0.5U and 1U of the enzyme concentration

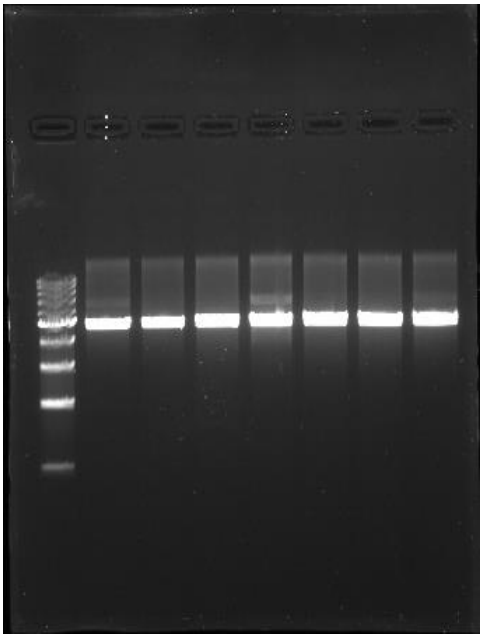

Fig 3– Denaturation gel for E.coli O157:H7 DNA

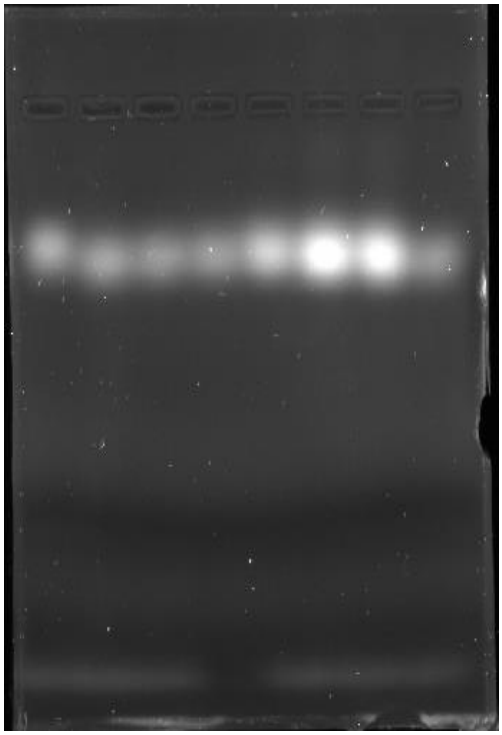

Fig 4 – Denaturation gel for pGLO plasmid DNA

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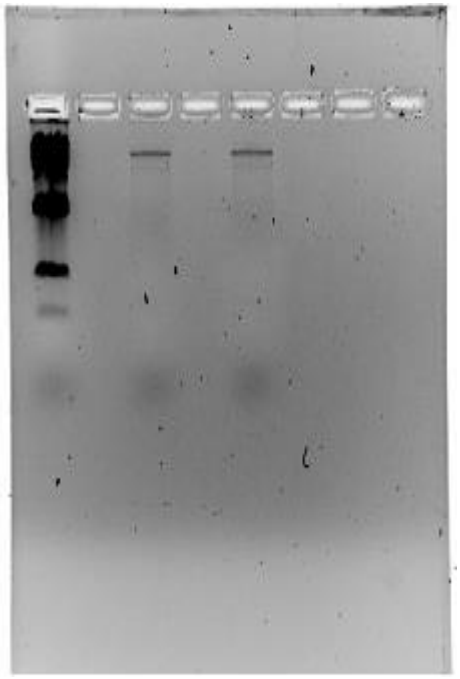

Fig 5 – Ligation of pGLO plasmid and the gene of interest obtained from the E.coli O157:H7 DNA

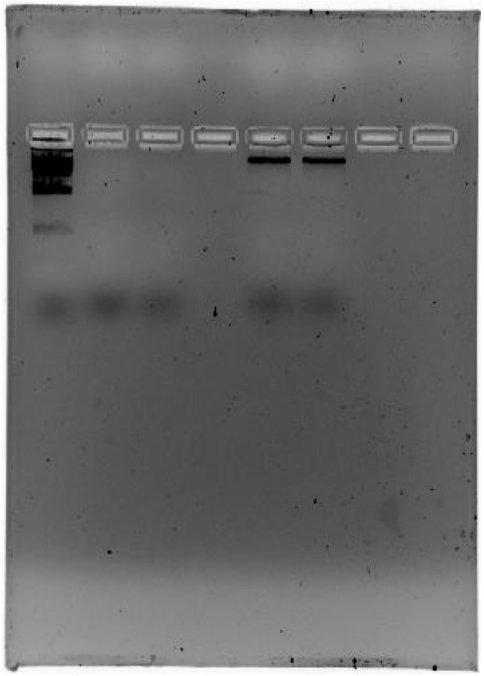

Fig 6 – fliglo samples obtained after ligation when run on the gel

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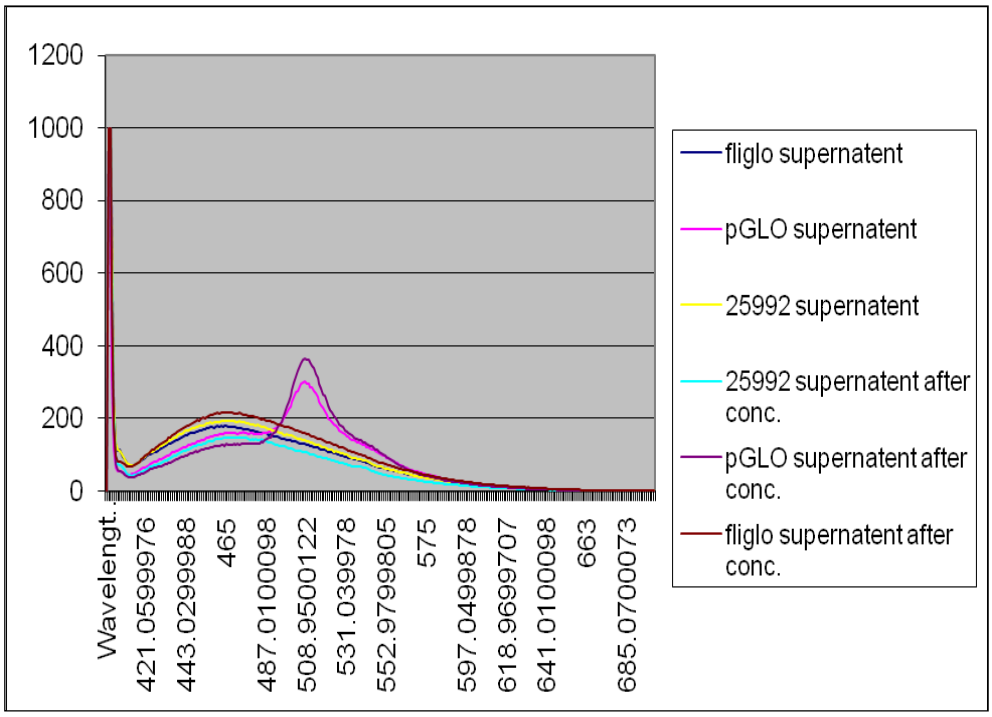

Fig 7-Graph obtained from the fluorometer