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Preview of the detection and identification of *Mycobacterium avium subsp. Paratuberculosis*

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Abstract

Mycobacteria are narrow, acid-fast bacterial cells. They are similar to other bacteria within the Gram-positive group in that their cell wall contains peptidoglycan, but their cell wall is distinguished in that it contains long-chain of fatty acids, called mycolic acids. The genus Mycobacterium contains fast-growing and slow-growing species. Mycobacterium avium subsp. Paratuberculosis (MAP) is classified among the slow growing species. MAP is recognised to be the cause of Johne's disease, which presents in the form of severe inflammation of the intestines of ruminant animals in addition to cattle, including sheep, goats and deer, as well as several kinds of non-ruminants, such as birds. The general symptoms of Johne's disease are cachexia and diarrhea. Infection of cattle causes significant economic losses due to decreased milk production, premature culling and increase in veterinary costs. MAP has also been suggested to cause Crohn's disease in human, which is characterised by a gastrointestinal disorder, where patients suffer from severe abdominal pain, diarrhoea, bleeding and bowel obstruction. The problem with paratuberculosis is that it is difficult to diagnose in its early stages as no symptoms appear in the beginning of the infection and by the time that general wasting in cattle and a decrease in milk production are detected, infection is at quite an advanced stage. The methods which used to detect MAP are Microscopic detection, Culture detection, Enzyme-linked immunosorbent assay (ELISA), Polymerase chain reaction (PCR) and Bacteriophage-based Techniques. However, there is a limitation to each technique. Therefore there are researchers working on developing a novel method to detect MAP using green fluorescent protein (GFP). The aim of this paper is to display the methods of detection and identification of Mycobacterium avium subsp. Paratuberculosis.

Key words: *Mycobacterium avium subsp. Paratuberculosis,* Johne's disease, Enzyme-linked immunosorbent assay, Polymerase chain reaction, Bacteriophage-based Techniques, green fluorescent protein.

Introduction and literature review

Mycobacteria are narrow, acid-fast bacterial cells. They are non-motile, obligate aerobes (Anon, 1999; Harris and Barletta, 2001; Ryan and Ray, 2004). Genus *Mycobacterium* is the unique genus in the family of Mycobacteriaceae, within the order Actinomycetales (Rastogi *et al*., 2001). Mycobacteria are similar to other bacteria within the Gram-positive group in that their cell wall contains peptidoglycan, but the difference is that the Mycobacterial peptidoglycan is composed of N-glycolylmuramic acid, rather than N-acetylmuramic acid. In addition, the mycobacterial cell wall is distinguished in that it contains long-chain of fatty acids, called mycolic acids. Mycolic acid constitutes a large percentage of the total cell wall mass (approx. 60%; Anon, 1999; Ryan and Ray, 2004) and the unique waxy nature of the mycobacterial cell wall gives these cells an ability to resist attack by certain chemicals used as sanitisers, such as chlorine. Further, comparing with other genera of bacteria, mycobacteria can resist some physical processes, for instance pasteurization and UV light (Grant and Rees, 2009).

The genus *Mycobacterium* contains fast-growing and slow-growing species. *Mycobacterium avium subsp. paratuberculosis* (*MAP*) is classified among the slow growing species (up to 18 weeks to form colonies when growing on solid media). *MAP* is capable of remain alive in soil or water for several months and even years i.e. can persist in a dormant state (Nacy and Buckley, 2008). Various species of mycobacteria are able to produce mycobactins, which vary according to their structure, such as the location of their fatty side chains. For instance, mycobactin A is produced by *Mycobacterium aurum*, mycobactin R is produced by *M*. *terrae*, mycobactin Fisproduced together with mycobactin H by *M*. *fortuitum*, and mycobactins M and N are produced by *M*. *marinum* (Snow and White, 1969). The main role of mycobactin is uptake of iron which is required for essential mycobacterial metabolic pathways (Rodriguez, 2006). However *MAP* is unable to produce mycobactin (Nacy and Buckley, 2008; Grant and Rees, 2009) and because of this the addition of a synthetic mycobactin J to culture media is necessary for cultivating of MAP *in vitro* (Grant and Rees, 2009).

Several species of Mycobacteria are known to be pathogens of humans and animals. *MAP* is recognised to be the cause of Johne's disease (also known as paratuberculosis; Manning and Collins, 2001; Anon, 2007),

which was discovered by Johne and Frothingham in 1895 (Harris and Barletta, 2001; Payeur, 2005; Manual, 2015) and presents in the form of severe inflammation of the intestines of ruminant animals (Harris and Barletta, 2001). The general symptoms of Johne's disease are cachexia and diarrhoea (Chiodini *et al*., 1984; Manning and Collins, 2001; Anon, 2007). Infection of cattle causes significant economic losses due to decreased milk production, premature culling and increase in veterinary costs. Paratuberculosis is common worldwide and infects many types of ruminant in addition to cattle, including sheep, goats and deer (Nacy and Buckley, 2008) as well as several kinds of non-ruminants, such as birds (Anon, 2007).

MAP has also been suggested to cause Crohn’s disease in human (Anon, 2007). This disease is characterised by a gastrointestinal disorder, where patients suffer from severe abdominal pain, diarrhoea, bleeding and bowel obstruction. Some researchers are concerned that Crohn's disease has been caused by patients consuming milk contaminated with MAP. It has appeared in pasteurised milk in some grocery stores, and also it may contaminate cheese made from this milk (Nacy and Buckley, 2008). As the symptoms of Johne’s disease are similar to that of Crohn’s disease, research was conducted to investigate the potential trophic transmission of MAP. It has been shown that raw milk and commercially pasteurised milk can be contaminated with MAP (Singh *et al*., 2010).

Disease progression and limitations of MAP detection methods

The problem with paratuberculosis is that it is difficult to diagnose in its early stages as no symptoms appear in the beginning of the infection and by the time that general wasting in cattle and a decrease in milk production are detected, infection is at quite an advanced stage. Because of this, it is difficult to eliminated MAP from individual animals in a herd and infected, asymptomatic animals can transfer pathogens to young animals through milk and manure. Consequently, this makes it impossible to eliminate the disease or protect livestock herds from *MAP* infection (Anon, 2007; Nacy and Buckley, 2008). Therefore, the detection of *MAP* in the early stages of infection is extremely necessary to help farmers eliminate Johne's disease.

Anon, 2012 reported that this disease has three stages of infection. In the first stage, infected animals do not show the symptoms of the disease and the bacteria are present only in limited places within the gut and do not cause significant damage. At this stage, the infected animals do not produce antibodies. In the second stage of this disease the bacteria multiply slowly and cause damage in the intestines. The infection

then spreads to other organs including the muscles, udder and womb, and the bacteria can be shed into the environment to infect other animals through faeces and milk. At this stage, the infected animals start to produce low levels of antibodies. Therefore, the antibody test will fail to detect many infectious animals. In the final stage, the number of bacteria being excreted by the animal continues to increase and the symptoms (e.g. weight loss and diarrhoea) start to appear. At this stage of the infection the production of antibodies by the infected animal increases and can be detected using ELISA-based assays (Anon, 2012).

Because faeces are readily available sample, they are commonly used to identify sub-clinical and clinical animals according to the levels of *MAP* shedding, so that faecal culture is one of the most common tests used for the diagnosis of paratuberculosis (Leite *et al*., 2013). However the methods which used to detect MAP in faeces are not very sensitive or specific, and the advantages and disadvantages of each of these is described below.

Microscopic detection

Diagnosis of Johne's disease is often performed based on detection of MAP in faecal samples by Ziehl–Nielsen (acid-fast) stain of a smear of faeces or intestinal mucosal tissue and subsequent examination under a microscope (Manual, 2015). Koch in 1882 used the staining method for the detection of acid-fast bacteria for the first time. The substance used as an indicator of the presence of mycobacteria is alkaline methylene blue (Sakula, 1983; Ellis and Zabrowarny, 1993). This stain was developed in the same year by Ehrlich, then by Ziehl in 1882 and 1883 (Ellis and Zabrowarny, 1993; Raoul, 2009). The limitations of this staining technique are that it does not differentiate between the species of mycobacteria. In other words, it does not confirm the presence of (for instance) *MAP* in the specimen, rather just indicates that a species of acid-fast bacteria is present. Microscopic detection also has low sensitivity and requires experience to confirm a negative result following the observation of many microscopic fields. Nevertheless the method is commonly used and paratuberculosis infection can be confirmed on microscopic test of a single faecal specimen (Gentry, 2005; Manual, 2015).

Culture detection

Detection of MAP to diagnose Johne's disease in infected animals is also performed by cultivating of faeces and tissues samples in solid media such as Herrold's Egg Yolk Medium (HEYM) or Middlebrook 7H10. This is considered to be the most definitive technique to identify *MAP* in clinical samples. Since faeces contain many microbes other than *MAP*, and their growth is faster than that of *MAP*, chemical decontamination

must be carried out before samples are cultured to eliminate these microbes. However, *MAP* is able to survive this process and it is possible to detect the presence of the organism by growth (Chiodini *et al*., 1984; Stabel, 1997; Manual, 2015). However, decontamination can impact on the survival of MAP as showed by Grant and Rowe (2004). They reported that treatment with 0.75% cetylpyridinium chloride (CPC) for 5 h at room temperature significantly reduced the survival of MAP.

Despite the fact that culture is possible after chemical decontamination, the two-four months required to grow MAP using conventional cultivation methods leads to delay diagnosis of Johne's disease. As a result, many researchers have tried to develop faster methods of detecting the disease (Colgrove *et al*., 1989). Culture media methods were improved by introducing indirect detection of growth in liquid culture. For instance, the BACTEC system was designed for the growth and detection of mycobacteria from human clinical specimens (Grant *et al*., 2003) which detected the release of radioactive CO_2 following the catabolism of radio-labelled palmitic acid by the growing mycobacteria. This system has now been replaced by the Mycobacteria Growth Indicator Tube (MGIT), which was introduced to the first time in the mid-1990s by Becton Dickinson (BD). MGIT is a modified Middlebrook 7H9 broth that produces a fluorescence to indicate bacterial growth (Pfyffer *et al*., 1997). In this system oxygen acts as an indicator to growth of mycobacteria, where when MGIT tubes are placed on an U.V. transilluminator (365 nm wavelength) an orange fluorescent glow is emitted, due to the consumption of oxygen (Grant *et al*., 2003). The significance of this technique is that more rapid detection of mycobacterial species can be achieved (Pfyffer *et al*., 1997; Abendano *et al*., 2012). However, the disadvantages of this method are that the procedure takes approximately 40 days and that chemical decontamination of the sample is still required which reduces sensitivity. In addition, the growth indicator is also not species-specific and therefore PCR is still required to confirm presence of MAP in a sample (Pfyffer *et al*., 1997; Kawaji *et al*., 2014; Grant and Rees, 2009). In a study for Kawaji *et al*. (2014), the researchers detected MAP in faecal samples by using the manual fluorescent MIGT culture system beside solid culture (Middlebrook 7H10 agar-based slants).At this experiment, the MGIT medium tubes were incubated at 37°C for 84 days and monitored manually once a week to capture an image of fluorescence from MGIT tubes. MGIT-positive tubes were sub-cultured onto Middlebrook 7H10 agar-based slants to confirm bacterial growth on solid media. Additionally, DNAs was extracted from MAP cells using the boiling method, and then tested by Quantitative PCR (qPCR) targeting the MAP-specific IS*900* element, to confirm the growth of MAP. This study found that the rate of MAP detection using the MGIT system was higher than that using 7H10 agar-based slants alone. Furthermore, the time to obtain a final result for faecal culture by the MGIT system was faster compared to culture on solid media, even though it still required 12 weeks. This study also showed that MAP was isolated from 43 of 61 faecal qPCR positive faeces.

Enzyme-linked immunosorbent assay (ELISA)

Tests have also been developed to diagnose paratuberculosis in a herd using immunological assays such as enzyme-linked immunosorbent assay (ELISA). This research relies on the detection of specific *M. paratuberculosis* antibodies in serum (Stabel, 1997). Colgrove *et al*. (1989) proved that the ELISA format was more sensitive than two other common serological tests; complement fixation (CF) and agar gel immunodiffusion (AGID). Several commercial absorbed ELISA kits have been provided and all these commercial ELISAs seem to be similar in their sensitivities and specificities. Some commercial kits are designed to test milk samples, while others have been designed for blood serum samples (Manual, 2015).

Despite the development of these ELISA kits, diagnosis of subclinical paratuberculosis still presents a huge problem for the control of Johne's disease. The reason of this is that infected animals generally do not produce measurable antibody titers in the early stages of disease (Stabel, 1997). The performance of different techniques for the diagnosis of paratuberculosis was examined by Paolicchi *et al*. (2003). In that study, 24 cows that were infected with MAP, but did not show symptoms of the paratuberculosis, were used. The researchers applied blood serim ELISA tests, beside other different methods (AGID, gamma interferon (yIFN), bacteriological cultures, PCR and direct examinations of smears using Ziehl–Neelsen's (ZN) stain) on faeces and milk samples. The finding of that study demonstrated that more than 40% of the experimentally infected animals were ELISA positive. They suggested that ELISA is a useful method for the diagnosis of paratuberculosis, but it is still ineffective in detecting subclinical infection in a herd in the beginning of disease.

Polymerase chain reaction (PCR)

PCR has the advantage of rapidity and it can be used to diagnose Johne's disease within about 3 days. However the efficiency of this technique can be influenced by the quality of nucleic acid samples, and the thick waxy MAP cell wall can make the extraction of DNA difficult (Leite *et al*., 2013) which reduces the sensitivity of any PCR-based method. In addition the presence of PCR inhibitors in faeces, including phytic acid and polysaccharides, makes this a major challenge when trying to detect MAP in faecal samples and may lead to false-negative results by inhibiting the amplification of DNA (Leite *et al*., 2013). Englund *et al*. (1999) detected MAP in 92 tissue (ileum, and lymph nodes) and faeces samples from cattle by using single PCR to confirm growth of MAP in primary bacterial cultures, and the amplification was performed with the primer p11 and p36, targeting IS*900* and primer 901a and 901c, targeting the IS*901*element also found in *M. avium* subsp. *avium*. Further, they used nested PCR which is a development of IS*900* element (it involves two sets of primers, used in two successive runs of polymerase chain reaction; the second set

intended to amplify a secondary target within the first run product) to analyze seventeen tissue samples, previously found MAP positive by microbiological culture. In this study, the nested PCR was carried out by using four primers were selected from the MAP specific insertion sequence IS*900*. It has been reported that, 58 of the 92 colonies were detected by IS900 PCR, while the IS901 PCR has not demonstrated in any samples, exhibiting that the colonies were not *M*. *avium* subsp. *avium* carrying the IS901 element. Additionally, ten of the samples were positive with nested PCR and seven were negative. Englund*et al*. (1999) concluded that the nested PCR could be a useful technique for the diagnosis of MAP, where its sensitivity and specificity higher than single PCR, but this sensitivity is still lower comparison to conventional culture methods.

Stabel and Bannantine (2005) developed a nested PCR assay to detect MAP in faecal samples. They used a unique element IS*Map02* that is present at six copies within the genome, and compared detection of this element with detection of the IS*900* element using both conventional and real-time PCR which uses the same principle of amplification as conventional PCR, but instead of looking for PCR products on a gel at the end of the reaction, the reaction is placed in to a real-time PCR machine that measures the increase in fluorescently labelled PCR product with a camera or detector (Anon, 2008) to estimate the sensitivity of these two methods. The researchers also examined the specificity of the IS*Map02* element using PCR of the DNA extracted from different isolates of mycobacteria including MAP. It was shown that less than 100 fg of DNA was required for the IS*Map02*-specific nested PCR protocol and 10 fg of DNA for the IS*900*-specific PCR protocol. The authors also reported that the sensitivity of both the IS*900* and the IS*Map02* element were improved up to 1 $\log_{10}$ by using real-time PCR. They also found that all samples were positive by both IS*900*-specific conventional and real-time PCRs, however One faecal sample from a cow that had clinical disease and high numbers of MAP resulted in a negative band on the gel after conventional PCR was performed; whereas the same sample was positive after the real-time method was performed. Additionally, one faecal sample from an infected cow that shown signs of paratuberculosis was negative for the *ISMap*02 element by both PCR tests, and the real-time PCR technique for the ISMap02 element was able to detect two animals that were negative by conventional method. Stabel and Bannantine (2005) concluded that the sensitivity and specificity of a PCR which developed with ISMap02 element provides an alternative or additional PCR assays for the detection of paratuberculosis.

Bacteriophage-based Techniques

A Phage-based test was used for the first time to detect mycobacteria in the 1960's (Rees and Botsaris, 2012). Goldman and Green (2015) indicate that using phage-based tests as a diagnostic method depends on specificity of the interaction between the virus and the host cell.

Bacteriophage-based rapid methods have been developed for detection of MAP in milk within 48h (Altic *et al*., 2007; Stanley *et al*., 2007; Grant and Rees, 2009; Goldman and Green, 2015). The use of phage to detect MAP improves the sensitivity compared to conventional culture methods because there is no need to use chemical decontamination of the milk sample prior to the assay (Botsaris *et al*., 2009; Grant and Rees, 2009). However the bacteriophage used does not infect MAP alone and therefore specificity has to be achieved by combination with other methods. Stanley *et al*. (2007) used the FASTPlaqueTB assay reagents to detect *MAP* cells within 48 h and used a PCR-based identification method to add specificity to this assay. Research by Foddai *et al*. (2011) applied a Peptide-Mediated Magnetic Separation-Phage Assay (PMMS-phage) to detect MAP in 44 bovine bulk tank milk samples; 25 fresh and 19 previously frozen at -70°C for several months BTM samples, and 39 faecal samples. In this study, the faecal samples were processed through the optimized PMS-phage assay and then resuspended in Middlebrook 7H9 broth supplemented with a nystatinoxacillin-aztreonam (NOA) to limit the growth of background microflora during the period incubation, whereas some of the BTM samples were cultured onto HEYM after the decontamination process with 0.75% hexadecylpyridinium chloride (HPC), and the other samples were cultured into Middlebrook 7H9 broth without prior chemical decontamination; to show the effect of the decontamination method on survival of MAP. Foddai *et al*. (2011) also performed Plaque-IS*900* PCR on PMS-phage assay-positive samples to confirm the presence of MAP. The results showed that 10 of the fresh BTM and 5 of the previously frozen BTM samples yielded plaques, and all the 10 positive fresh milk and 4 of the 5 positive frozen milk samples were confirmed to contain MAP with IS*900* PCR. The authors also reported that the chemical decontamination before culture has adverse effects on the viability of MAP leading to reduced detection sensitivity. They also found that plaques were obtained for 27 faecal specimens, and 20 of these samples were positive with IS*900* PCR. The researchers noted that faecal samples would be the optimal to process via the PMS-phage assay compared to milk samples.

Following the use of phage to detect MAP in milk, Swift *et al*. (2013) reported that PMMS combined with a phage-based detection method could be used to detect *MAP* present in the blood of infected cattle within 48 h and that there was a good correlation with both milk and blood ELISA results indicating that the method could probably be used as a diagnostic technique for Johne's disease.

However, despite the development of a range of different detection methods, the problems connected with each of these for the detection of MAP means that further methods are required. Therefore some researchers aim to develop a novel method to detect MAP using green fluorescent protein (GFP).

Green fluorescent protein (GFP)

The discovery and development of the green fluorescent protein (GFP) which was isolated from jellyfish (*Aequoreaaequorea* or *Aequoreavictoria*) as a biological cell label for the first time in the early 1960s resulted in Marty Chalfie, Roger Tsien and Osamu Shimomura being awarded the Nobel Prize in Chemistry in 2008 (Anon, 2008; Zimmer, 2009). *Aequoreavictoria* Green Fluorescent Protein (avGFP) is an unusual protein, and its crystal structure shows that it contains of a β-can which comprises an 11-stranded β-sheet polypeptide wrapped into a pseudosymmetric cylinder. There is strong visible wavelength 4-(p-hydroxybenzylidene)-5-imidazolidinone fluorophore which is produced autocatalytically from the protein's own amino acids placed at the centre of the avGFPβ-can. GFP contains 238 amino acids and the fluorophore is formed spontaneously at position 65-67 from serine (Ser), tyrosine (Tyr) and glycine (Gly). The characteristic of all the GFP proteins is that the protein structure promotes the conversion of this tripeptide into the chromophore (Prasher *et al*., 1992; Heim *et al*., 1994; Ehrenberg, 2008; Frommer *et al*., 2009).

Discovery of the fluorescent protein has encouraged the researchers to develop many applications for the study of cell biology. For instance, imaging gene expression *in situ* and creating of biosensors that are based on genetically modified fluorescent proteins (Frommer *et al*., 2009). Currently GFP is used widely because its success as protein fusion partner and the fact that a variety of different proteins have been isolated which emit fluorescence at different wavelengths of light. Schmelcher *et al*. (2010) designed 18 different fluorescent proteins for a rapid multiple detection of *Listeria* strains. These proteins were created by fusing the C-terminal cell wall binding domains (CBDs) to fluorescent marker proteins of various colours. The researchers bound the fusion proteins to 26 *Listeria* strains. They used fusions with GFP with other differently coloured fluorescent proteins such as cyan fluorescent protein (CFP) and RedStar protein (RS) for the construction of reporter fusions with CBDs, and then used fluorescence microscopy to image the bacterial cells labelled with different colours. It was shown that *Listeria* strains were selected according to the binding specificity of each fluorescent protein-CBD. The authors concluded that CBDs offer high specificity, and fluorescent CBD fusion proteins can detect *Listeria* strains in contaminated milk or cheese in short time and with a high specificity through direct microscopy. This study demonstrated that Gfp fusions could be used to specifically identify bacterial cell types using fluorescent microscopy.

More recently Swift (2014) developed a novel fluorescent proteins to detect MAP that can be an alternative to non-specific acid-fast staining. This protein was designed by fusing GFP protein to peptides that have been shown to bind specifically to MAP cells to produce fluorescent MAP-specific label. Swift (2014) used microscopic visualisation techniques to detect individual MAP cells that had been labelled with

this protein, and showed that they did not bind to other non-pathogenic *Mycobacterium smegmatis* cells present in the same sample.

Creating new proteins by fusing fluorescent proteins to other proteins that can bind to Mycobacteria

Bacteriophage endolysins are peptidoglycan hydrolases, where they lyse the host at the end of its multiplication phase. These enzymes containing C-terminal cell wall-binding domains (CBDs) which specifically target the enzymes to their substrate in the bacterial cell envelope, and because of this they used in development of novel diagnostic tools for rapid detection of pathogenic bacteria (Schmelcher *et al*., 2011). Fusion proteins with novel and optimize properties were created by Schmelcher *et al*. (2011) for labelling and lysis of *Listeria* using phage endolysins. In this study, different CBD domains targeting various *Listeria* cell surfaces with high specificity, and for this reason, some researchers aim to apply this study with mycobateria phage lysins for detect MAP strains to diagnose paratuberculosis.

Related experiments conducted by the author of this paper

CaCl2 transformation Experiment

10 ml Nutrient Broth (NB) (OXOID) were Inoculated with cells from an overnight culture of E. coli BL21 DE3 pLys9 to A600nm = 0.05, and then were grown with shaking at 37°C to A600nm = 0.35. Next, the culture was diluted with fresh NB (pre-warmed to 37°C) and was grown again with shaking at 37°C to A600nm = 0.35. The cells were pelleted in a cooled rotor (5°C) at 3.5 K x g for 10 min, then the pellets were resuspended in 5 ml ice cold 100 mM MgCl2 and incubated on ice for 1 h. The cells were pelleted as before and then the pellets were resuspended in 1 ml ice cold 100 mM CaCl2 and incubate on overnight packed in ice. 250 µl of cells were transferred to a sterile Eppendorf tubes. 2 µl DNA of four different kinds of *E*. *coli* Top10 that designed by Swift (2014) (N-aMptD GFP, N-MP3 GFP, GFP C-aMptD & GFP C-MP3) (The original peptide: aMP (Stratmann *et al*., 2002); aMptD and aMpR (Stratmann *et al*., 2006)) were added to cells and mixed well. Additional to +ve and –ve control were made, using test DNA (1µl) for +ve control. The Eppendorf tubes were stood on ice without disturbance or mixing for 1 hour. Then tubes were shocked by heat at 42°C for 3 min, again without disturbing, and then were returned to ice for 10 min. The whole transformation mixture was added to 5 ml of pre-warmed (37°C) NB and incubate with shaking for 30 min.3 x 100 µl samples were plated out in NA supplemented by amp. 100µg ml^{-1}an overnight at 37°C, and then the remaining cells were pelleted at 4000 xg in room temperature for 10 min. Next, the remaining cells were resuspended in 0.3 ml NB, and then plated (3 x 0.1 ml) an overnight at 37°C.

Result

Transformed cells have been formed (Figure 1). These cells were plated in NA plates that supplemented by amp 100µg $ml^{-1}$and Isopropyl-beta-D-thiogalactoside (IPTG) 0.5 mM using Patch Plate method. The plates were incubated overnight at 30°C. Then, the colonies have been excited by using blue light. However, no fluorescence was appeared (figure 2).

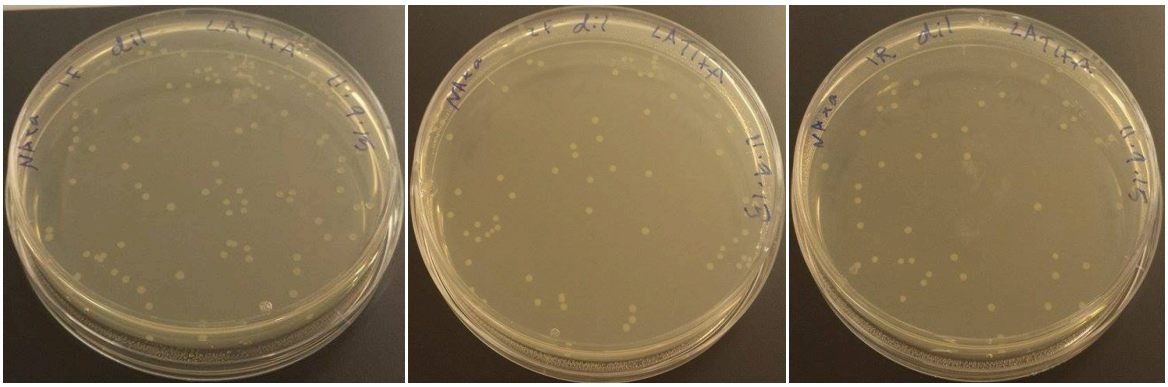

Figure 1. Transformed cells.

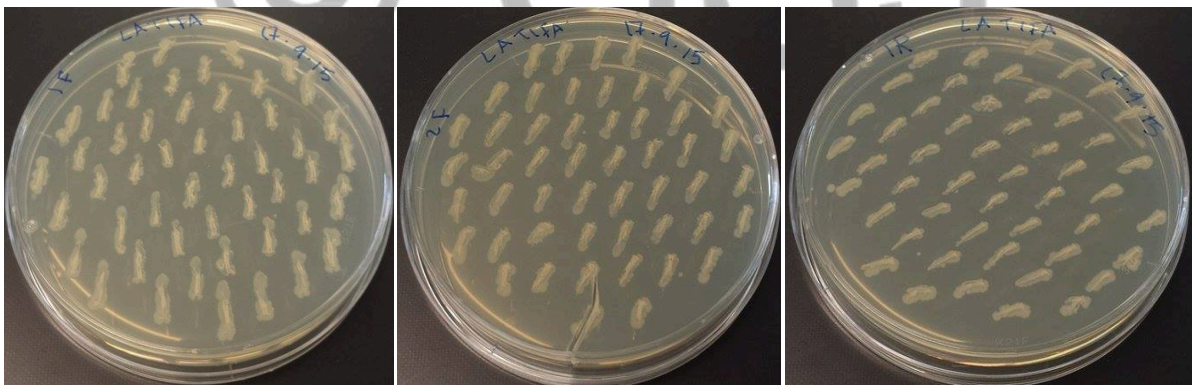

Figure 2. Transformed cells grown in Patch Plates.

The transformed cells that were grown in Patch Plates were plated in LB broth supplemented by amp. 100µg $ml^{-1}$and IPTG 0.5 mM using 96 Well Assay Plate (Black Plate), and then incubated overnight at 37. Next, the fluorescence was measured in Tecan.

No fluorescence was shown, and because of this the LB broth was replaced using MRD media. The results display the fluorescence in various transformed cells (table 1). These cells were sub-cultured in LB plates supplemented by amp. 100 µg ml-1 and IPTG 0.5 mM (figure 3,4).

Table 1. Fluorescence measurement using Tecan.

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	274	15441	273	1856	293	1674	277	1995	271	33185	265	24749
B	271	2374	268	3125	272	1247	272	4515	268	1181	263	2184
C	270	6003	279	10830	270	14162	264	6117	266	1576	265	37803
D	270	15084	266	6720	261	1781	260	869	263	1163	263	1950
E	268	2259	268	2837	262	25224	257	24693	264	1187	265	16241
F	266	1808	267	1716	264	27377	265	2348	264	1290	265	44169
G	265	1934	268	2017	267	1494	266	2074	262	31300	257	2474
H	5868	1624	269	809	11988	27866	266	802	6332	1391	255	762

2-4=1F, 6-8=2F, 10-12=1R

(1H, 5H, 9H=No amp.; 4H, 8H, 12H=No cells).

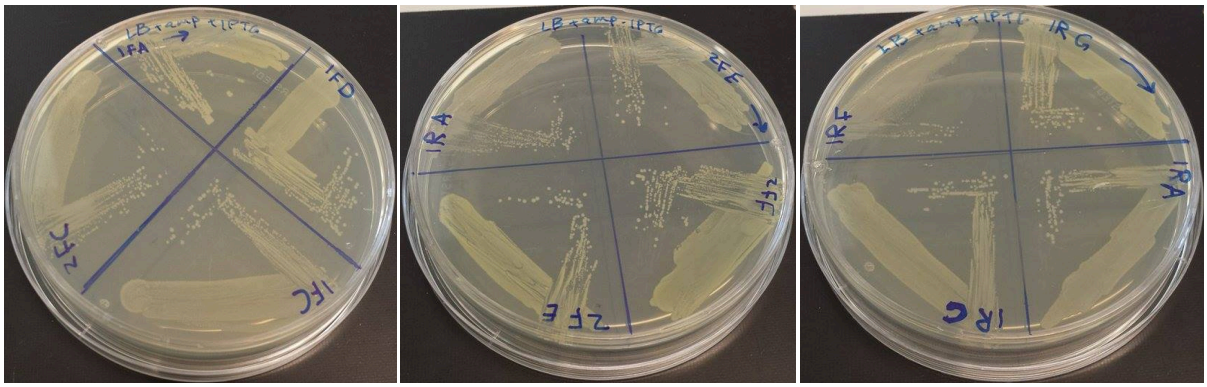

Figure 3. Transformed cells appear fluorescence using blue light.

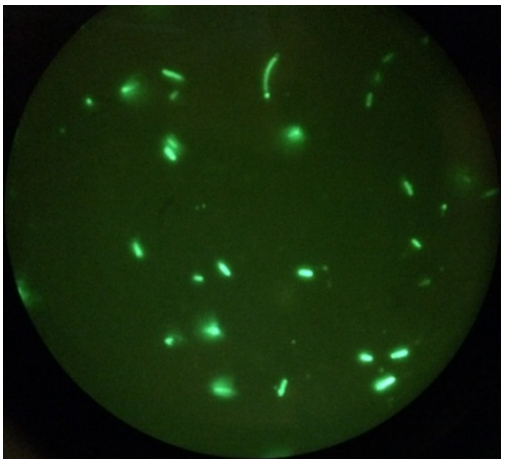

Figure 4. Green fluorescent cells under microscope.

The plasmids of transformed cells which include GFP were isolated using QIAprep® Spin Miniprep Kit, and the length of DNAs were measured using gel electrophoresis method as the following:

0.8% agarose gel was prepared using 1X tris, acetic acid and EDTA buffer (TAE), and contains 0.5 µg/ml ethidium bromide. The gel was placed in gel box, and the running buffer was added until the gel was completely submerged. 5µl DNA size standards (1-Kb ladder) were transferred to the first well. Then, each of the DNA samples (5 µl DNA and 1µlLoading Dye 6X PROMEGA, G19OA) was transferred to wells. Next, the power was turned on and the voltage was set to 70 to about 50 min. The gel was slid off the tray onto the UV light box of the photodocumentation System to photograph it and save an image in electronic format (figure 5).

Figure 5. DNA bands for transformed cells (1FA, 1FC, 1FD, 2FE, 2FF, 2FE-, 2FC, 1RA, 1RC, 1RA-, 1RF and 1RG).

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