PCR-RDB Assay for Detection of *Vibrio cholerae*

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CERTIFICATE

This is to certify that the dissertation entitle "PCR-RDB assay for detection of *Vibrio cholerae*" is the bonafide record of research work done by Mr Foong Sui Yun during the period from June 2004 to March 2005 under our supervision.

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1.0 ABSTRACT

A colorimetric detection method has been designed and optimized by immobilizing a specific DNA probe onto Biodyne C membrane and hybridize it with heat denatured biotin labeled PCR amplicon to detect the presence of the *hemM* gene of *Vibrio cholerae.* Therefore it can be used to replaced agarose gel electrophoresis and conventional culture method which are very low in sensitivity for diagnosis of cholera. This method was refered to as PCR-RDB assay. Optimization of the PCR-Rerverse Dot Blot assay include hybridization temperature, amount of PCR amplicon, timing of the assay, optimizing of biotin incorporation in PCR amplicon, dilution of streptavidin-alkaline phosphatase conjugate used and development of a control positive for the assay. PCR-RDB assay can be used in detection of biological or environmental samples to help in control of cholera cases in high incidence area.

1.0 ABSTRAK

Suatu kaedah pengesanan kalorimetrik telah direka dan dioptimumkan dengan melekatkan probe DNA yang spesifik pada membrane Biodyne C dan dihibridasikan dengan produk PCR berlabel biotin yang dipecahkan kepada rantai tunggal dengan cara pemanasan. Kaedah ini boleh mengesan kehadiran *Vibrio cholerae* dengan kehadiran *hemM* gen. Maka ia boleh digunakan untuk menggantikan elektroforesis agarose gel dan keadah penkulturan semasa yang tidak sensitif pada pengesanan pathogen ini. Optimasasi asai PCR-RDB merangkumi suhu hibridasasi, jumlah PCR amplicon, tempoh asai, optimisasi kemasukan biotin pada PCR amplicon yang baru terhasil, pencairan konjugat streptavidin-alkalin phosphatase dan pembangunan asai positif kontrol untuk memastikan keputusan asai PCR-RDB ini. Asai PCR-RDB boleh digunakan pada sampel biologikal atau alam sekitar membantu dalam pengawalan kes-kes kolera yang tempat yang kejadiannya tinggi.

2. INTRODUCTION

2.1 Vibrio Cholerae

Diseases causing vibrios give three major syndromes of clinical illness which include gastroenteritis, wound infections, and septicemia. Many cases of this pathogens is associated with gastroenteritis are under recognized because vibrios are not readily identified in routine stool cultures. Etiologies of vibrio gastroenteritis are foodborne and highly associated with consumption of raw or undercooked shellfish and unhygienic handling of food and lifestyle is also predisposition factors (Nicholas, 2000).

Vibrios are gram-negative, curved, rod-shaped, and microaerophilia bacteria that are natural inhabitants of marine environment. It is know that the transmission of vibrios infections is primarily through consumption of raw or undercooked shellfish or exposure of wounds to warm seawater. Other source include water, ice, rice, food and beverages from street vendors and food left out at room temperature for several hours as comment by Blake et al (1980), St Louis et al (1990), Tauxe et al (1988) and Pavia et al (1987). *Vibrio* infection is self-limited gastroenteritis however wound infections and primary septicemia may also occur. Another factor due to its under recognized because most clinical laboratories do not routinely use the selective medium for vibrios, thiosulfate-citrate-bile salts-sucrose (TCBS) agar for processing of stool specimens unless they specifically requested to do so especially in Gulf Coast states as reported by Marano et al in year 2000. There are at least twelve pathogenic *Vibrio* species recognized to cause illness in human which includes *Vibrio cholerae,* followed by *Vibrio vulnificus,* and *Vibrio parahaemolyticus.*

V. cholerae O1 is the primary causative agent of the disease cholera according to a paper written by Morris et al in year 1985. Its clinical manifestation include profuse watery diarrhea, vomiting, and muscle cramps which eventually leads to dehydrating diarrheal illness with substantial loss of fluid and electrolytes. It had been reported on occasion that stool volumes may approach 1 liter/hour and confirmed by Pierce et al (1969) and Hirschhorn et al (1968). *V cholerae* O1 is further subdivided into El Tor (commonest biotype) or Classical biotype. It can also be divided by serotype like Inaba, Ogawa, or Hikokima and by toxin production can be toxigenic or nontoxigenic (Greenough et al, 1995).

Even vibrio is mostly related to unhygienic practice and foodhandling does not rule out its occurrence in developed countries. Example, in United States remains low incidences of vibrios cases because of access to safe drinking water and good sanitation but residence travel to area of high incidence of cholera in developing countries may risk a higher chance in contact with the pathogens. This is most likely due to an increasing number of Americans traveling abroad. US health care facilities were not adequately prepared to diagnose and treat cholera as reported by Besser et al (1994).

Diagnosis of this disease via conventional culture required about 24 to 72 hours whereas if the laboratory use PCR it can usually be faster. Culture methods consist of sample transportation medium in alkaline peptone water (APW), TCBS, and slide agglutination with specific antisera that can be used to detect serotype and assay for cholera toxin production. Norazah et al (Malaysian Medical Association) in year 2001 attempt to detect *Vibrio cholerae* O1 from 80 aquatic environment samples following an outbreak of cholera.

Designed primers for *ctx* gene and polymerase chain reaction and conventional culture methods was use to detect the present of the pathogens. Recovery of *Vibrio cholerae* by culture method was poor which only detected one positive case. However polymerase chain reaction have detected eight positive samples and confirmed it derived from single source when DNA finger printing by pulsed-field gel electrophoresis is used consistence with outbreak.

Thus regardless how developed a country, a simple but efficient test must be able to test not just the presence of vibrios in stool or biological samples but also in environment samples as well so that the cholera incidence can be detected earlier and controlled. Following this more focus can be directed toward the treatment and complication arises from the disease. With the completion of the full genomic sequences of *Vibrio cholerae,* a specific primer pairs can be designed and used in the detection of the pathogens with a very popular molecular tool, polymerase chain reaction.

2.2 HemM (LolB)

According to previous research done earlier by Ravichandran et al (2000), *hemM* gene is chosen for the new and faster detection of *Vibrio cholerae*. *HemM* is actually a non-virulent gene and its sequences can be found in Genbank with the accession number of AF227752. *HemM* gene is 633bp in length and capable of detecting all the *V. cholerae* strains as it is a consensus region that is coded for outer membrane lipoprotein LolB.

Other PCR assays developed for *V. cholerae* based on virulence gene *ctx* and *hemolysin* does not identify nontoxigenic infectious *Vibrio cholerae* O1. The primer VHMF and VHA-AS5 design from Ravichandran (2000) was used

because of it superiority in detection of all the *Vibrio cholerae* O1 regardless of its virulence. However the *hemM* PCR amplicon is present for *V. furnisii,V. mimicus* and *Shigella flexineri*. *V. furnisii* produce as a non-specific PCR product of 700bp, *V. mimicus* 200bp and *Shigella flexineri* 950bp. Hopefully with the shifting of a new detection platform this problem will be solved.

2.3 Polymerase Chain Reaction

Polymerase Chain Reaction is a tools used by scientist worldwide to amplify a specific sequence in eukaryotic or prokaryotic cell such as in microorganism or in human to million times. This technique was developed by Cetus Corporation in the year 1984. Previously laboratory technician detected pathogenic or causatic agents like bacteria either by culture methods or antibody result from the immune response from immune competent individual. This type of detection focus on the evidence that the living bacteria were once there, however with PCR, the diagnostic can focus on the pathogenic bacteria as well (Alcamo et al, 1996).

With PCR it is possible to reproduce DNA in tubes so it can now be fragment, composition determination, structural changes, modify and even map it genes. Its principles have been applied to detecting infectious disease, linking criminals to crime, cancer and genetic disease screening, and maintaining public health by identifying pathogens in the environment so step can be taken.

In PCR, specific primers are used to produce many copies of double stranded targeted region present in the DNA template. Thus by PCR amplification, the presence of the interested region in DNA template can be

detected and viewed by agarose electrophoresis. As much as million of copies of PCR amplicons can be synthesized in few hours in PCR.

In PCR multiplication of a DNA molecule, 5 main components are required: specific primer that will bind to a unique region in the DNA template thus initiating the DNA replication process; DNA polymerase or *Taq* polymerase, an enzyme derived from *Thermus aquaticus*, that will direct DNA replication process but unable to start the process; mixture of nucleotides which contain dATP, dGTP, dCTP, and dTTP, that is the substrate for the double stranded amplify DNA; and buffers, which is essentials for the maintenance of the whole PCR reaction. *Taq* polymerase from bacteria *Thermus aquaticus* is very heat stabile thus can withstand the constant heat of 95°C for 30 minutes. Some of these enzymes have half-life over 40 minutes at 95°C like *Taq* DNA polymerase from company Fermentas.

Generally, PCR reaction are repetition of 3 steps for specific duplication of the interested target DNA molecule performed in a PCR machine. In the first step, reaction mixture contain double stranded DNA template is denatured at 95°C to break the hydrogen bonds that hold two DNA strand together and to unwind the DNA molecule. Next, the temperature is lowered to facilitate a specific binding of primers flanking the complementary site in the DNA template. These primers "mark" the starting and ending of the duplicating region. Lastly DNA polymerase in presence of Mg2+ ion will complex with primer and the DNA template and fill in the blank area by incorporating the substrate nucleotides so single stranded DNA is transform into double stranded DNA. Continuations of these steps will resulted in mass reproduction of double stranded DNA amplicon that contain the specific region from the DNA template.

However the ability to reproduce a specific DNA amplicon means nothing if there is no method to certain that is the double stranded DNA that we want. Hence agarose gel electrophoresis containing Ethidium bromide is generally used as it intercalates between double stranded DNA and glows under UV light. Usage of a molecular marker helps us to determine the size of the PCR amplicon.

2.4 Visualization by Agarose Gel

Agarose is purified from linear galactan hydrocolloid which is isolated from agar or recovered from agar bearing marine algae. Initially use for protein analysis and purification, it is later developed for DNA work.

Double stranded DNA is visualized in agarose gel in the presence of ethidium bromide and UV light. The double stranded DNA in the agarose gel will fluoresce as orange color.

2.5 DNA Probes

Sol Spiegelman and Edward Hall disovered that single stranded DNA will bind to a complementary strand of RNA and form a double stranded DNA-RNA molecule. Scientist started using principle of DNA-DNA match and research the possibility of using a DNA strand to recognize complementary DNA molecule in a mixture of DNA molecule. They succeeded in discovering that the DNA probe could be used to detect DNA targets.

A DNA probe is a small (10 bases or greater than 10,000 bases), single-stranded molecule of DNA that recognizes and bind to complementary segment of DNA on large DNA molecule. The fact that Adenine (A) specifically binds to

Thymine (T) and Guanine (G) to Cytosine (C) thus DNA probe obtain its high specific complementary binding. Other important feature that must be present in designing probe is that the probe should not hybridize to other non-specific nucleic acid molecules or other non 100% complementary region. Once hybridization has formed the binding must remain stable.

The strength of binding between AT is less compared to GC as AT have only two hydrogen bonds whereas GC is linked by three hydrogen bonds. This hydrogen bonds can be broken by heating over 90°C, submerge in medium with higher pH or in organic compounds such as urea or formaldehyde. Like in PCR reaction this will cause the denaturation of the double stranded DNA. Adjustment of the temperature, pH or conditions of the salts will cause the single stranded DNA to anneal or bind to the same complimentary region to form back the double stranded DNA. This process is term hybridization of DNA.

This binding of the specific probe to the DNA molecule alone is not sufficient if we cannot "see" thus some minor modification on the DNA probe is required. Sometimes DNA probes are labeled with radioactive isotope, biotin, DIG, streptravidin, and other molecule that are crucial for visualization. When labeled DNA probes has bind to segment of the DNA molecule than using method like fluorescence, colorimetric and isotope. Their presence can be viewed by naked eye and recorded permanently.

In early 1980s, the optimism generated by the use of DNA probes was counterbalanced by the problem of insufficient DNA available in sample to perform a reliable and reproducible test. It has become a limitation to all scientists until the birth of polymerase chain reaction (PCR). Thus we need only to focus on the detection of these DNA probe or the synthesize PCR

amplicon. Southern blot is routinely used to detect the presence of these DNA probe binds to PCR amplicon. Agarose gel that contain the run PCR amplicon samples were transfer using the southern blot to nylon membrane. DNA denatured to single stranded followed exposure to UV light will crosslink and immobilize it on the nylon membrane. Specific DNA probe will then search out and binds to the specific segment in the single stranded denature PCR amplicon and later detect the specific binding using enzyme, flourescein, or radioisotope methods.

Problem of amount of PCR amplicon and DNA probe problem have been explored now we start a great leap which will help us understand more of DNA molecule and its application for the benefit of man or womankind.

2.6 *Literature Review*

The knowledge that DNA molecule can amplify to larger amount of the same fragment using PCR reaction, DNA probe specifically binds to a segment of DNA molecule and PCR amplicon can be labeled by a marker to be detect have challenge the modern scientist to develop another powerful detection and diagnostic tools. This new tools will not just benefit the medical but flourish in other industrial aspect as well; Oligonucleotide-based technique for detection of pathogens that can be divided into PCR and hybridization.

PCR amplicon is amplified by a set of specific primers which flank the region of interest. Other similar methods that perform continue PCR reaction so that different segment can be amplified such as in multiplex PCR. Regardless of what modification at the end, the specific bands were visualized in the agarose gel stained with ethidium bromide and compare with known patterns. Other methods include hybridization of the labeled PCR molecule to immobilize oligonucleotides. Both have the advantage of screening for presence of specific pathogens or detecting the biodiversity of the organism or virus in samples. It is reported that hybridization of multiple specific probe increase confidence over single probe in presence of pathogen-derived DNA molecule. But this does not necessarily establish the hybridization of multiple probes to the same molecule in the population of targets. Different from these, PCR amplicon is a contribution of two or more highly specific primers binding specifically to the same DNA template. Combine with hybridization of DNA probe it result in a very strong dual recognition for the specific regions.

Lockley et al in year 1997 immobilized poly-T primers on nylon membrane that resemble dipstick with another PCR primer present in PCR

reaction mix. PCR reaction was run with both the soluble primer and immobilizes primer on nylon membrane with no change to the parameter when both soluble primers were used. During PCR amplification, both primers anneal to template strands and extending it. Newly synthesize PCR amplicon will act as template strands to the next reaction. Finally soluble and immobilize primers both are elongated and incorporated with DIG due to the dTTP is DIG labeled. The extended immobilize poly-T primer on dipstick was later detected using the colorimetric methods using NBT as substrate. A round dot is visualized for positive result and no dot was seen on the dipstick for negative samples. Lockley et al uses UV light to crosslink the poly-T primer to the nylon membrane Hybond-N instead of the bake over the oven method. UV crosslinking methods immobilize forming a covalent bond between the NH_2 groups of the membrane and the light-activated thymine groups of the nucleic acid. Lockley et al choice of using UV crosslinking is good as Nierzwicki-Bauer et al (1990) showed that UV-immobilized DNA at 254nm with an exposure of 120miliJoules/cm^2 using UV Stratalinker 1800 in nylon membrane has increase in sensitivity in detection. However oligonucleotides immobilized in nylon membrane by UV-immobilization, DNA may be lost as repeated stripping and heterologous reprobings and when use nitrocellulose membrane yielding worst result. Nierzwicki-Bauer et al (1990) however notes that baking method which involves baking in vacuo for two hours at 80°C is less sensitive even it retain the DNA molecule after repeated stripping. So UV-immobilize DNA in nylon membrane is stable and sensitive in detection if the same blot is not over or frequently restripped and reprobe. Importantly Lockley et al (1997) uses two different types of poly-T oligonucleotide primer with allele-specific dotted on the same

strip. Each dot give positive result independently thus the strip method was able to extend when the template DNA was available as template. On the other hand, Lockley et al (1997) only immobilize a single primer of a PCR pair was thus extension of and detection of multiple spots will suffer same problems as multiplex PCR. Problems arise from unwanted interaction among the non-immobilized primers and result in accumulation of mispriming of PCR amplicon. This eventually reduced amplification caused by the interaction of primers with templates and PCR amplicon in unintended ways despite computer assisted design. In addition to that, newly synthesized soluble PCR amplicon is wasted and was not involved in the final detection step. So some reagents are loss in this method. Zhang et al (1991) reported that covalent linkage provides an increased sensitivity of the assay compared with method used by Lockley et al (1997) which utilize T-tail-based attachment. Usage of spacer between the amino group and the oligonucleotide had no effect on the binding efficiency to membranes.

Boles et al however have developed a method know as Bridge Technology™. Unlike conventional PCR where all PCR amplification occurs with fluid phase primers and later hybridize to solid phase. Bridge Technology™ amplification occurs on solid surface and all amplified amplicon remains covalently bound in specific pixels on the medium. Sets of specific PCR primer pairs were immobilized via their 5' ends on the surface of the solid support. Each of these pixels has multiple copies of single PCR pair (either positively charge or negatively charge). In solution phase, target DNA anneals to one of the immobilized primer (for example the positive charge part) and is extended in the PCR first cycle. Newly synthesize strand is covalently attached

to the support via the primer linkage. Second cycle of the PCR involve the immobilization of the first cycle amplicon now act as template for extension by an immobilized primer of opposite polarity (negative charge). The second cycle PCR product "bridge" the two primers with both strands covalently attached to the support. In third cycle, both immobilized strands is denatured to single strand and acts as template for new primer extension thus providing mechanism for exponential signal growth. These newly synthesized PCR amplicon can be labeled by fluorescence, radioisotope, DIG, biotin by including labeled dNTP so it can be visualized later. This novel method has four main benefits. First, single Bridge test can detect many hundred of genetic markers. Second, there is no interference from primer artifacts and unequal amplification efficiency between different primer sets. Third, cross contamination of samples with amplified DNA does not happen as amplified DNA remains bound to the solid phase during and after amplification. Finally, detection methods by using optical can monitor amplification is real time and reducing assay time and providing reliable method for quantifying input target copy number. According to Onodera et al (2002) that this method is limited by the necessity for acrylamide polymerization thus utility of the technique for the preparation of large assays of primer pairs is difficult (Figure 2.6.1).

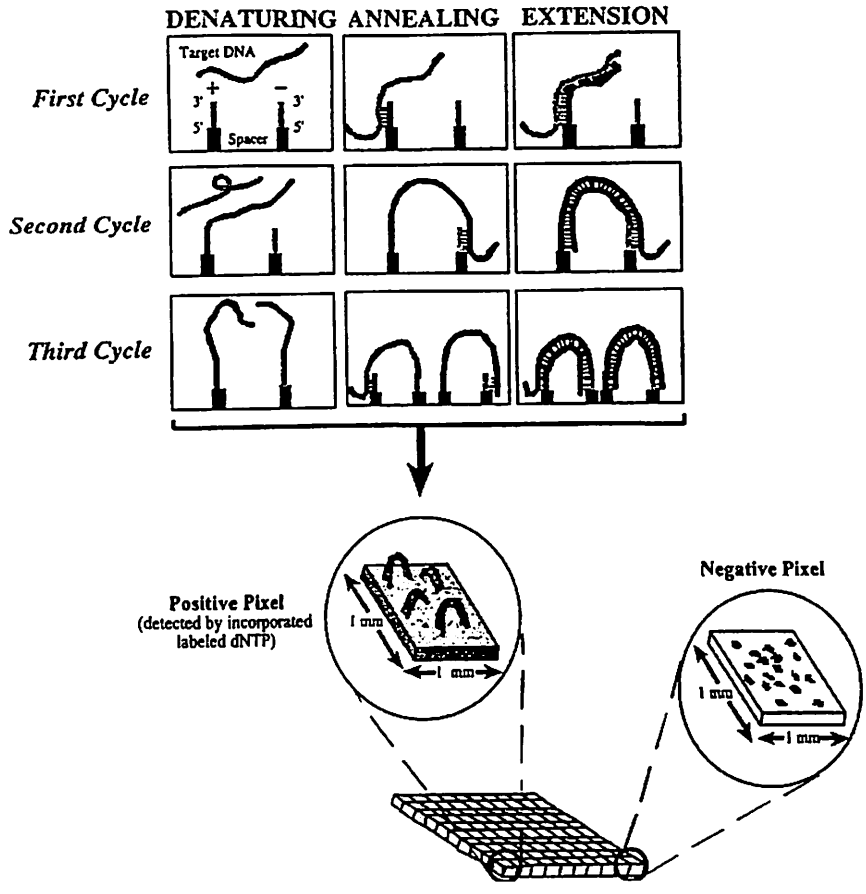

Figure 2.6.1: Illustration showing the principal of the Bridge Technology™

All the previous mention method is not very good. A good diagnostic tool must have criteria like ease of use, technical difficulty, time to obtain the result, and cost of the assay must not too expensive for the consumer. Godfroid et al in 2003 have proposed the use of PCR-Reverse Line Blot; a typing method underscores the genomic heterogeneity of *Borrelia valaisiana* species. Specific DNA probe with amino end is immobilized onto nylon membrane using a pump mechanism that can deliver control amount of the DNA capture probe. After PCR amplification of the *Borrelia* strains DNA, the PCR amplicon is denature and hybridize onto the specific capture probe. A detection probe carried a multifork-like structure on its 5' end with eight biotin group was designed to bind on the single stranded PCR amplicon. It is later visualized using alkaline

phosphatase and substrates NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate). Godfroid et al (2003) have successfully showed that the *Borrelia* strains can be correctly differentiated using the PCR-RLBA (PCR-reverse line blot assay). It show strong correlation with the PCR-RFLP based on *Mse*I restriction pattern on PCR-amplified 5S-23S intergenic region (PCR-based restriction fragment length polymorphism). Besides that PCR-RLBA is easily interpretate compare to PCR-RFLP which need to cross check with the know patterns. PCR-RLBA can be performed in less than 3 hrs and does not use toxic chemical agents or radioisotopes and electrophoresis apparatus. Thus this method can be easily implemented in any laboratories that have a PCR machine. Sadly the probe that carried multifork-like structure that contains eight biotin groups will increase the cost thus the accessibility for the public.

In 2003 year, Hoelzle et al have developed another diagnostic PCR assay for detection of *Mycoplasma suis*. PCR amplicon is later processed by southern blot and dot blot analysis. Hoelzle et al uses 1.8kb *Eco*RI-REA fragment and 782bp PCR amplicon from purified *Mycoplasma* were used as probe. Purified DNA as much as 100-500ng were labeled with digoxigenin-11-dUTP by a random oligonucleotide primer method supply by Roche. Hoelzle et al also blotted denatured PCR amplicon onto a nylon membrane Hybond-N by alkaline capillary blotting according to method suggested by Sambrook and Russell et al, 2001. Prehybridization is done at 37°C for half an hour and hybridization at 37°C for 8 to 12 hours. Anti-digoxigenin Fab fragment conjugated with alkaline phosphatase is used in the next step of immunodection. Although the PCR dot blot showed some false negative as compare to culture, it does give positive result on one sample. Maybe due to

the nature of the DNA extracted from blood and other parameter discuss in the paper the test seem to be not very suitable for diagnostic work. However the detection method using PCR dot blot does seem to be simple only the prolong period of hybridization is the only setback especially when this assay suppose to perform in routine lab that may required an overnight hybridization.

O'Connor et al in 2000 have published a PCR but modified detection method for *Listeria* and *Listeria monocytogenes* in food which is called probe membrane-based assay. O'Connor et al designed PCR primer pair which will amplify the 16S to 23S. Few oligonucleotide capture probe was designed within that region. These capture probe is added with amino group at 5' end so that it can form an amine bond between the carboxyl groups present on the Biodyne C nylon membrane and the 5' capture probe. An equipment call Biodot liquid dispensing machine, xyz 3000 Dispensing platform is used to line a 1mm wide 1ul/cm line on the Biodyne C membrane. The immobilized capture probe is then hybridized with heat denature biotinylated PCR amplicon at 95°C for 10 minutes. The hybridization step is done for 60 minutes at 37°C on shaking platform. This is when the single stranded heat denatured biotinylated PCR amplicon bind to the complementary region with the capture probe. Streptavidin-alkaline phosphatase conjugate is used for the colorimetric development of the strips. It is found that a strip can detect using up to 4 different probe with 1 control positive. The assay developed can detect as low as 1 CFU inoculated into 25ml of milk following overnight culture. It is a very sensitive test.

A reverse line blot (RLB) was initially developed from reverse dot blot assay for diagnosis of sickle cell anemia (Gubbel et al, 1999). Both techniques