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Review Article

Novel PCR-ELISA Technique as a Good Substitute in Molecular Assay

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Abstract

Due to the spread of infectious diseases, the existence of a rapid and sensitive detection method is necessary today. Polymerase chain reaction-enzyme linked immunosorbent assay (PCR-ELISA) is a simple manner for detection of microorganism. For example, bacteria, viruses, fungi and others based on nucleic acid sequence. A large number of samples can be screened by this technique simultaneously, so it is not time consuming and is a quick manner. The high sensitivity and specificity of PCR-ELISA make it a powerful technique by simple laboratory facilities. As a result it can be an excellent substituted manner for analysis and detection in different various fields.

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Introduction

The Introduction of PCR-ELISA and its applications

PCR-ELISA is containing of PCR and ELISA techniques that allows to detection of nucleic acid instead of protein[1].With this assay, we can determine quantity the PCR product directly after immobilization with biotinylated DNA on a microplate.PCR is a molecular technique that was invented by American chemist, Kary Mullis, in 1984 [2]. However, principle of replicate DNA by primers was discovered by Gobind Khorana in 1968 [3]. PCR can copy the Large number of DNA copies and this technique is used in many laboratories for detection to days [4]. PCR technique just can replicate a piece of DNA, for there development a complementary manner is necessary for detection of PCR product [5, 6]. It is Including of gel electrophoresis [7], fluorescent in situ hybridization (FISH), southern blot and others. ELISA is a pathobiological technique which formed from reaction between antibody and antigen. This is a semi-quantitative technique if with the color intensity can be discovered the presence or absence of sample [8].

Novel PCR-ELISA technique is an immunological method to quantify the PCR product directly after immobilization of biotinylated DNA on a microplate, is used for clinical and food stuffs. It was included of a new way for detection and identification of pathogenic bacteria in the environment , clinical and food samples [9]. Until now, many different of bacteria, viruses, and fungi have been detected by this assay. Despite these advantages PCR-ELISA has not been used as a quick, easy and non-expensive detection method in clinical laboratories because it is not as well as introduced until now. PCR-ELISA is a more accurate diagnostic test in compare with other common molecular and serological tests [10].

PCR-ELISA is based on interaction of between DIG-labeled DNA sequencing and anti-DIG antibody. By using of probe in this technique, if target DNA is wrong copied, probe cannot connect to it and the answer will be negative [11]. After sampling, a specific part of the gene is selected and primers designed for it [12]. Amplification was carried out by digoxigenin-11-dUTP (DIG-dUTP) nucleotide. Probe is labelled with biotin at its 5'end. Preferred probe complementary in the middle of the gene as it can increase the specificity of the connection to complementary sequence. At the first, streptavidin which has high affinity to biotin is coated in microplate. Double strand DNA is singled by heat shock and is added to microplate. Complementary sequence is connected to probe and extra material is washed with PBST. In the final step, anti-DIG antibody is added and optimal density is measured by spectrophotometry [13]. This manner is quick, easy and can be getting the exact result at maximum 4 hours, without needed to advance laboratory and professional person. This method is safe and its color is non- mutagenic [10]. PCR-ELISA is a semi-quantitative technique [8] and although the result of that, is not exact amount of sample in comparison to real-time PCR but real-time PCR is more expensive than PCR-ELISA. So, when has not required the exact amount of samples, PCR-ELISA is a suitable alternative (Figure 1).

PCR -ELISA status in molecular diagnostics

Comparison of PCR-ELISA with the other diagnostic techniques

Since the introduction of this method, various molecular techniques have been designed until now. Due to the detection of products labeled to DIG with PCR-ELISA, makes this method a more sensitive than agarose gel electrophoresis analysis. Because in this way products are analyzed by colorimetric method, thus the risk of toxicity of

color materials and DNA pollution is reduced. As this detection uses gene-specific probes for detection, the specificity of the assay is too high. Another advantage of this method is large-scale screening with standard laboratory equipment. The compared with conventional PCR methods, the analytical time of PCR-ELISA is shorter and this is an important advantage of this method. Due to its low cost, if study does not require very high sensitivity, PCR-ELISA can be a good option instead of quantitative PCR (qPCR) (semi-quantitative method) (Table 1) [14].

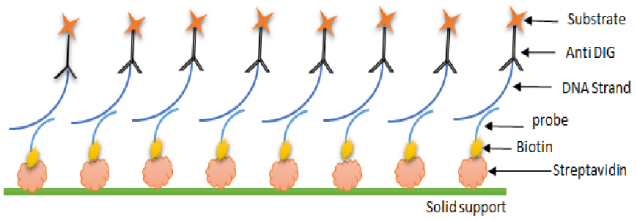

Figure 1. Detection of biotinylated DNA using an anti-DIG-peroxidase conjugate with substrate ABTS to forma blue-green color reaction that is both visible and measured using a spectrophotometer

This method can be applied for identification and quantification. These techniques have been used in different fields including of genetic [15], pathobiology [16], medicine, and pharmacy. The using of these methods can be pointed to genetic disorder, hereditary cancer, solid tumors, neoplasia, hematopathology, and infection disease [17]. When the PCR was invented, great evolution has been taken place in this concept and many techniques have been made from polymerase chain reaction, contains of generation PCR, restriction site multiplex PCR, allele specific PCR, real-time PCR, PT-PCR and some else. Today as distinguish a lot of disease, it is very important in the field of medicine that there is a quick and not expensive technique. A lot of techniques can be used for detection, needed to cultivating sample or biochemist reaction. This manner is time consuming, need to proficiency and as well expensive [18]. PCR-ELISA is a simple and an exact manner for using in different laboratory.

In this technique do not require to culture bacteria and a piece of DNA can be replicated by usual PCR, after that, ELISA is performed to receive desired result. By using of multi kits can recognize types of microorganism in short time. PCR-ELISA does not require using dangerous and carcinogenic material that have used in many of techniques, including of methylene blue which used in electrophoresis agaros gel. As result a laboratory technician can be worked in safe place. Using of specific primers to amplify a piece of gene and specific probe should be designed. The probe can increase the sensitivity and accuracy of PCR-ELISA assay [19]. A lot of laboratory tests, need to high expertise people so training the professional and deployment them in laboratory has the high cost, as FISH technique because of high specificity for detection of chromosomes need a proficient. But PCR-ELISA does not require to high expertise and have been carried out by simple expert. Some bacteria especially in a family that has same symptoms and this make difficult to their detection. High specificity and sensitivity of PCR-ELISA resolve this problem, too. This method does not need laboratory facilities and can be performed in simple laboratory with simple equipment. As viral detection, PCR-ELISA is 10 fold more sensitive than nested-PCR. The only major problem of nested PCR is a great risk of contamination. If no internal control is applied and using gel electrophoresis in nested-PCR is not sensitive for very low viral loads and considered as false negative results [20]. The only limitation of this method is detection of bacteria in the genus compared to multiplex PCR and gel electrophoresis [10]. Reverse transcription polymerase chain reaction ELISA (RT-PCR-ELISA) can be used for detection of RNA virus as that this technique is more sensitive than conventional RT-PCR. Conventional RT-PCR cannot handle large number of samples [21]. The sensitivity of fluorescent antibody test (FAT) has been reduced to detected autolyzed tissue samples. PCR-ELISA can be an alternative diagnostic test when the samples are unsuitable for use in FAT and also a supplementary test to FAT [22].

PCR -ELISA technique and comparison with the real-time PCR

PCR-ELISA is a semi-quantitative technique; in other word the exact amount of sample cannot be measured by PCR-ELISA.

Table 1. Comparison 3 molecular technique, conventional PCR, PCR-ELISA and real-time PCR.

Comparison	Convectional PCR	PCR-ELISA	Real-time PCR
Equipment	Standard laboratory	Standard laboratory	Require florescence detection instrument
Cost	Low	Low	Costly
Quantitative	Not-quantitative	Semi-quantitative	Quantitative
Specificity	Low	High	Moderate
Carcinogen material	Methylene blue	Not carcinogen	Florescence material

Indeed what comes of this method is the presence of microorganisms. Changing color shows the presence of gene and the color intensity shows amount of microorganisms which were detected. However, the exact amount of samples can be measured by real-time PCR [23]. In real-time PCR, exponential phase can be detected at the first step of reaction but in manual PCR the detection was performed in last phase. In PCR reaction, in any phase, reactants are used, and amount of them is became less.

So the obtained result in any phase is different and indicates quantity amount in least step of PCR. In PCR-ELISA, PCR product has be used after the end of PCR product. However as using SYBR Green in real-time PCR and it can connect to any kind of nucleotide sequence (nonspecific sequence), the obtained result is not really exact. Using methylene blue is carcinogen too. On the other hand florescence reporter although provide the exact and specific result but it is very expensive. According to these, we can conclude that PCR-ELISA is less expensive than real-time PCR [24]. Targeting in many methods is the presence or absent of sample and amount of them is not very important. So PCR-ELISA is more economical and a good alternative to real-time PCR [11]. To detection of RNA virus cannot afford the equipment, probes and expertise to perform real-time RT-PCR-ELISA based assays. So as sensitivity of real-time RT-PCR-ELISA and RT-PCR-ELISA is similarity, RT-PCR-ELISA is a good alternative (Table 1) [21].

The advantages of PCR-ELISA technique

Semi-quantitative techniques

PCR-ELISA is a semi-quantitative technique [25].Just with appearance of color, it can be understood that there is the intended sample or not. The color intensity shows the amount of approximately. In many experiments, understanding the presence of microorganism is enough and has not need to exact amount of this. Although real-time PCR determine the exact number but it is not necessary in a lot of times and the amount of approximately is enough. On the other hand real-time PCR is an expensive method [26].

High sensitivity

At many of researches that have been carried out in the field of PCR-ELISA, amount of sensitivity has been measured and detected. By preparing different serial dilution from samples and do PCR-ELISA sensitivity of that reported to the extent nano and sometimes picogram per µl [27]. However, the sensitivity of PCR and ELISA alone is so less from that. The sensitivity of PCR-ELISA for detection of E.coli O157:H7, is 1.08 pg/µl but the LOD of this bacteria by electrochemical impedance spectroscopy (eis) is 10-103 CFU/ml [28]. Monitoring transmission of *Wuchereria bancrofti* showed that PCR-ELISA is more sensitive than the traditional dissection techniques [29].

Detection in a short time

PCR-ELISA is not time consuming technique. The required time period for that is about 4 hours [9]. This time in comparison with other techniques for example culture of bacteria that need to remain in shaker incubator overnight is too short. Since the time is very important in medical and detection, PCR-ELISA can be a good alternative to another time consuming manner [30]. The comparison with real-time PCR and ELISA indicate that ELISA is found to be less time consuming and easier to perform than real-time PCR [31].

The distinction between germs with similar clinical symptoms (specificity)

To design PCR-ELISA for any samples, specific gene is detected. Any microorganism has some DNA sequence that is specific for that and differentiation it from another microorganism. So if two microorganisms for example two bacteria have same symptom, as DNA specific sequence is different, by PCR-ELISA can differentiate them together. There are a lot of techniques that show same result for different organism, especially bacteria from same family. Also, there is a lot of virus with same symptom like Hepatitis A and Hepatitis E [32]. The existence of a quick and exact manner for differentiation of them together is very important. Since in this technique used specific primers and probe if the DNA sequence is not an intended sequence the PCR reaction has not been carried out. On the other hand if DNA sequence is replicated by non-specific primers, specific probe never can be matched to this. As a result the reaction cannot be done and the result is negative [33].

Lack of carcinogenicity

There are a lot of materials in laboratory that can be extremely carcinogenic and create risk for laboratory personals. One of this is methylene blue that has been used in electrophoresis agarose gel, florescence materials that are used by real-time PCR and also tetramethylethylenediamine (TEMED) that used for preparation of acrylamide gel. Methods and materials that are used in PCR-ELISA are completely safe and has not any danger for laboratory personals [10].

Lower cost compared with the real-time PCR technique

Although real-time PCR shows the exact quantity of product but it is very expensive method. Materials that are used for make color are so expensive while PCR-ELISA is very simple and in comparison with real-time PCR is lower cost. This technique proved to be economical [34].

Usable in the laboratory with the least possible

PCR is performed by thermal cycler that find in any laboratory. In other hand ELISA kit is provided easily. So materials and methods that are useful in PCR-ELISA are simple and available in any laboratory. This technique can be used in simple laboratory with the facilities. PCR-ELISA has great potential of utilization in many laboratories [33].

Simultaneously detection of a large number of samples

According to numbers of wells in ELISA kit, it can be carried out for detection of several samples together, simultaneously. These samples can be different and create multi kit or they are same. This can reduce the time assay, too [35].

Limit of detection nucleotide sequences by PCR-ELISA

As mentioned, PCR-ELISA is extremely sensitive technique. The sensitivity of PCR-ELISA assay was 1.06×102 and $1.0^6\times10^3$ CFU/ml for pure cultures [36]. In many researches that have been carried out about PCR-ELISA, sensitivity of this was measured by making serial dilution from genome of samples. The detection limit of *Schistosoma* was 1.3 fg [8]. The sensitivity of PCR-ELISA for *E. coli* O157:H7 and *Shigella* was calculated as 1.08 pg/µl and 1.56 pg/µl, respectively [37]. Also, RT-PCR-ELISA could detect as little as 0.1 ng/µl HAV or HEV [32]. So the

lowest was femtogram per µl that reported PCR-ELISA can be detected.

The method is easy and does not require high expertise

As using simple manner in PCR-ELISA; it does not need to high expertise. A laboratory technician with simple knowledge can be carried out it. As a result, this manner does not need to employment expertise with high salaries [38].

Applications of PCR-ELISA

Detection and diagnosis

With the advent of PCR-ELISA assay, advantages and its semi-quantitative ability, a number of various studies were begun in this field. The Use of this assay as a rapid diagnostic method with semi-quantitative ability has been reported in many articles. For example, detection and identification of cancer cells, viruses, fungi, bacteria and their toxins [14].

Quantitative monitoring

For the presence or absence of a particular substance and determination of its concentration, PCR-ELISA can be used for quantitative monitoring as a quick indication assay. For example, this assay can be used for monitoring of cytomegalovirus infection in bone marrow transplant recipients and quantitative monitoring of *Leishmania* parasite in livestock [14].

Microorganism that detected by PCR-ELISA

Trypanosomes

Detection of Trypanosomes using PCR-ELISA was studied in 2009, by Cabrera *et al*. In this research, *Trypanosoma congolense* and *Trypanosoma vivax* were detected by three specific probes. They chose 18s ribosomal gene and used nested PCR. Nested PCR increased the sensitivity of PCR-ELISA to range of fg of target DNA. Also they compared with the sensitivity of PCR-ELISA and PCR-RFLP and proved that PCR-ELISA is more sensitivity than PCR-RFLP [39].

Hepatitis A virus and E

Takh *et al*., in 2011 detected *hepatitis A virus* and *E* by duplex RT-PCR-ELISA. Specific primers and probe for HAV designed from HAV 5′ noncoding region (NCR) and for HEV targeted to the well-conserved ORF2/ORF3 overlapping region. These two viruses have same clinical symptoms and duplex RT-PCR developed to detect simultaneously HAV and HEV.As virus has RNA, they have to use RT-PCR. Compression with duplex RT-PCR-ELISA for detection of HAV and HEV showed that this manner is more sensitive than RT-PCR and dot-blot hybridization. As real-time RT-PCR is very useful technique but it is expensive and need to high purity of DNA/RNA[32].

Papilloma virus

Papilloma virus in cervical cancer was detected by using PCR-ELISA by Raji *et al*., in 2011. They changed PCR-ELISA to utilize in this study. PCR-ELISA has two steps, PCR reaction and then does ELISA with PCR product. In this study has been made two step modifications in a phase. The name of this kind of PCR-ELISA has been DIAPOPs (detection of immobilized amplified product in a one phase system) [40].

Human immunodeficiency virus type I (HIV-1)

In a study in 2013 in Iran, PCR–ELISA was performed for quantification of HIV-1 with extracted DNA of thirty seropositive and twenty seronegative individuals. The specificity and sensitivity were 95% and 96.7%, respectively. Then this assay was compared with nested-PCR. As a result, PCR–ELISA was 10 fold more sensitive than nested-PCR [41].

Respiratory tract pathogens

Puppe *et al*., was researched about detection of 19 respiratory tract pathogens by PCR-ELISA. Culture techniques are expensive, low sensitivity and time consuming preferred to utilization molecular techniques. By m-RT-PCR-ELISA assay can be detected most of the non-colonizing organisms of the upper respiratory tract with one m-RT-PCR protocol and it can differentiate such pandemic infections agents from others [42].

Schistosoma infection

Detection of *Schistosoma* infection in feces was studied by Gomes *et al*., using of PCR-ELISA in 2010. For detection of *Schistosoma* infection usually use antibody bet as it cannot distinguish between current infection, past infection and cross reactivity, molecular tools should be considered. The PCR-ELISA showed that *Schistosoma*, could be detected 1.3 fg of genomic DNA which is equivalent to less than the DNA found in a single cell [8].

Coliforms in water samples

Kuo *et al*., detected coliforms in water samples by PCR-ELISA in 2010. Coliforms include of *Escherichia coli*, *Citrobacter* spp., *Enterobacter aerogenes* and *Klebsiella* spp. Target gene in this research was 16srDNA. They achieve LOD of 5 CFU/100 ml by PCR-ELISA that is superior to standard set have been performed by most countries (6-10 CFU/100 ml) [9].

Klebsiella pneumonia

Amani *et al*., detected *Klebsiella pneumoniae* by PCR-ELISA in 2015. *Klebsiella pneumoniae* is from Enterobacteriaceae family. 16srRNA was the target gene as it is an excellent phylogenetic marker. Specificity of PCR-ELISA was detected by genome replication of other members of this family. But they have not acceptable reply. The sensitivity of PCR-ELISA for detection of *K. pneumonia* was estimated 0.62 ng [43].

Salmonella typhi

Mousavi *et al*., detected *Salmonella enterca* serotype *Typhi* by PCR-ELISA in 2006. Standard methods for this study are time consuming (4-5days) but nucleic acid amplification was provided great sensitivity and specificity. The rfbE gene (CDP-tyvelose epimerase) was selected as target. Sensitivity of PCR-ELISA assay for detection of *Salmonella typhi* is 2.5 pg. Detection of *Salmonella* by bacteriological methods is time consuming (5-11days), so as PCR-ELISA is faster than that, it could management of out breaks better. Before detection of PCR product have been done by agarose gel electrophoresis but ELISA as 100-fold more sensitive than that [44].

Escherichia coli

Amani *et al*., studied targeting Shiga toxin 1 and 2 for detection of *E. coli* O157:H7 and *Shigella dysenteriea* in 2015. There are a lot of methods that have been used for detecting Shiga toxin such as culture, serological and molecular methods as RPLA, real-time PCR, and hybridization. All of these manners in compare to PCR-ELISA are so time consuming, quite costly and have limitation in handling many samples simultaneously. In the other hands molecular technique as PCR or real-time PCR are better

than microbiological techniques but for detecting PCR product must be used gel electrophoresis with carcinogen ethidium bromide. Real-time PCR is 100% specific and high sensitive but it is too expensive and need to expert. The sensitivity of PCR-ELISA for detection of Shiga toxin in *Shigella dysenteriae* and *E. coli* O157:H7 was 1.56 pg/µl and 1.08 pg/µl, respectively [37].

Vibrio cholera O1

Mousavi *et al*., in 2006 evaluated screening of toxigenic *vibrio cholera* O1 by PCR-ELISA. *Vibrio* is agent of cholera that is a pandemic illness, so if there is a quick technique that can be detected a large number of samples at the same time should be so benefit. Quick detection of *V. cholera* is extremely important [45]. *V. cholera* O1 was screened based on ctxB gene by PCR-ELISA. The sensitivity of that for ctxB, was estimated 0.5 pg/µl [46].

Fungal infections

Fungal infections with high morbidity and mortality have been increased in recent years. Antigen detection and molecular techniques can be carried out for rapid diagnostic assays. The compared with molecular methods based on gel electrophoresis, PCR-ELISA is more sensitive than these methods and multiple samples can be assayed in parallel [47].

Conclusion

Overall, PCR-ELISA is a simple manner for detection of microorganism, including bacteria, viruses, fungi and others based on nucleic acid sequence. A large number of samples can be screened by this technique simultaneously, so it is not time consuming and is a quick manner. The high sensitivity and specificity of PCR-ELISA make it a powerful technique by simple laboratory facilities. As a result, it can be used as an excellent substituted manner for analysis and detection in different various fields.

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