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Synthetic and Positive Constructs: A New Approach in Molecular Detection

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ABSTRACT

Genome-based and molecular identification of pathogens is a common and standard method for various microorganisms that are critical for controlling human diseases. In this field, preparation of standard genome/sequence is very important for setting up experiments and using it for molecular detection. For this prospect, microbial enrichment using its culture is necessary and for the most dangerous pathogens, laboratories with a high level of biological safety are very essential. Furthermore, the lack of access to some pathogenic genomes or standard strains in many countries is a major challenge for an accurate diagnosis. One intelligent and scientific strategy to solve this problem is using synthetic or artificial positive control constructs, which are utilized to ensure designed primers, probes, signal amplification and other characters of reaction works. This study reviews the design and development of positive control constructs for accurate and standard detection of dangerous pathogens for use in the manufacture and development of molecular diagnostic kits.

Keywords: Detection, Construct, Pathogen, Diagnostic kit

INTRODUCTION

The term synthetic construct refers to chimerical and hybrid plasmids that contain a definite sequence of DNA, designed and synthesized for particular purposes [1]. One of the applications of these constructs is diagnosing microorganisms like bacteria, viruses and fungi that may cause infections and diseases in humans, animals, or plants [2,3]. Today, bacterial and viral infections are one of the critical problems for global health. There are many methods to diagnose microorganisms including culture-based, serological, and molecular methods. These techniques have changed over time, leading to an increase in the rate and accuracy of diagnosis. Among these techniques, molecular methods are usually preferred because of reliability and accuracy [4, 5]. One of the significant challenges in molecular methods is accessibility to native microorganisms or genomic materials. For example, some pathogenic bacteria or viruses may not be found in some regions or have not been seen before, like the new coronavirus [6]. Therefore this is a critical issue for countries to have special tools for the detection of these pathogens even if they were not seen before in the related regions. Synthetic constructs could be designed according to the database information for each microorganism to simulate and develop diagnosis reactions. Also, these constructs are able to use as a positive control in molecular detection kits [7-9].

Critical Role of the Diagnosis of Microbial Infections

Various pathogenic microorganisms such as smallpox, diphtheria, cholera, tuberculosis, typhus, *etc.* can cause dangerous outbreaks and complicated diseases [10-12]. Also, the accurate detection of pathogens leads to suitable therapeutic methods. Generally, if the infectious agent is correctly detected the following steps to limit the spreading will be controlled significantly [13,14]. There are several methods for the diagnosis of microbes that differ from each other in the basic reactions. The cultured-based method, which is the oldest method in this field, was used widely in the past for the identification of bacteria or fungi [15]. This method is really dangerous for laboratory staff, but it is time-consuming. Also, some bacteria grow slowly or do not grow in laboratory conditions. Therefore, these approaches are usually not preferred to detect the pathogens, because of the risk factor of infectious diseases and the laboratory requirements. On the other hand, serological methods like enzyme-immune sorbent assay (ELISA), complement

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fixation (CF), and immune fluorescence antibody (IFA) assay are widely used for microbial agent detection. These methods especially act based on antibody-antigen reactions [16,17]. Today, the serological methods can be used for different aims in clinical laboratories and are useful but time-consuming and expensive in new cases compared to molecular methods. These methods are usually common in laboratories, however, their accuracy is lower than molecular methods. Furthermore, some of these serological methods can be combined with other methods, for example, the ELISA-PCR method, which has been reported for respiratory tract pathogens and is more efficient in comparison to individual methods [18,19].

On the other hand, the invention of polymerase chain reaction revolutionized all fields of molecular biology. After the invention of thermocycler, molecular approaches grew rapidly in the detection of organisms [20]. Molecular methods are more precise than others because they detect specific areas in the genomic regions of organisms. Nowadays, due to the speed and reliability of diagnostic tests, these methods are usually preferred to other methods. These methods include PCR-based methods, isothermal amplification technique, and the DNA microarray [21-25]. The PCR-based methods including conventional PCR, nested-PCR, multiplex PCR and quantitative PCR, are common and general molecular assays because of simplicity, and they are used to detect infectious and genetic diseases, as well as criminal issues [26-29].

One of the critical challenges in molecular methods is accessibility to the native microorganisms or genomic materials. Therefore, this is an important issue for the countries to have special tools to detect infection even without access to them before the outbreak. The second major concern is the bio-safety levels because some microorganisms are highly infectious and so, the risk of danger is comparatively high for the laboratory staff. Accordingly, it is necessary to have special laboratory conditions for working with these agents [30-32]. The laboratories with high bio-safety levels are rare in most countries due to economic costs. In this field, using synthetic control constructs is a smart process to solve these problems. These constructs could be useful for programming and developing molecular detection methods. As mentioned previously, some microorganisms may not be found in some areas so detecting them can be an important challenge. To solve this problem, using synthetic constructs is recommended because they can be designed for any known organism. Thus, any organism whose genomic information is recorded in databases can be selected as a target for the design of artificial structures in order to create a positive control for amplification [33-36].

The Artificial Sequence Design

Currently, the lack of a positive control sample is one of the main difficulties in the diagnosis of bacterial and viral pathogens. For this, the artificial genome and sequence design is an alternative approach for manufacture of molecular detection kits. These constructs contain specific genomic sequences of different pathogens. Designing specific primers and amplifying the desired sequences for synthetic vectors allows them to be used as a positive control in an amplification reaction. Because the sequence in the vector is similar to the pathogen, designed primers for the vector can similarly detect the pathogen in the experiment samples [37-42]. However, in this process, false-positive results are possible because the plasmid vectors are aerosol [43]. A routine PCR assay produces 10^9 copies of the target sequence and if aerosol contamination occurs, even the smallest aerosol will contain as many as 10^6 amplification products [44]. To solve this problem, some changes should be made in the vector sequence to make differences in the amplicon size of the vector, as well as the amplified region in the original pathogen. The genomic region of the organism is essential to apply the reaction in molecular methods. The existence of a synthetic construct that contains some specific sequences for inaccessible pathogens is vital. By using these vectors and the designed primers, a variety of molecular diagnosis methods could be performed according to the sequence properties [45]. Also, the reaction can be optimized by each molecular approach to ensure that the amplification has appropriately been performed. Moreover, the specificity and sensitivity of the designed primers can be considered in the reaction by these constructs using the plasmid copy number [33-36].

These vectors can be designed and used in a variety of molecular diagnostic methods and are suitable for the aimed method. All molecular detection methods require the

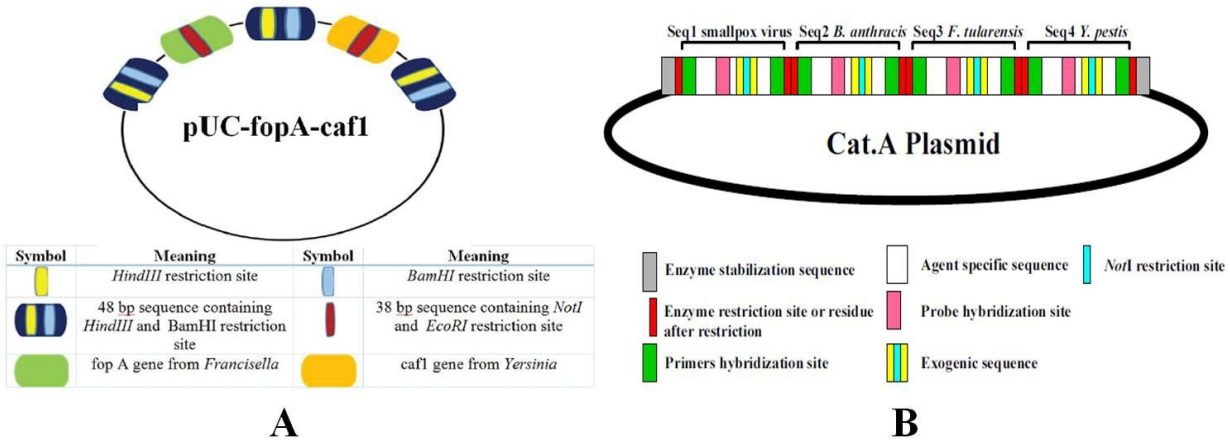

Fig. 1. Schematic presentation of two artificial constructs designed for molecular detection of (A) *Francisella*/*Yersinia* [1] and (B) *Smallpox*/ *B. anterasis*/ *Francisella*/*Yersinia* [38].

genome sequence of the organism for identification. Also, these vectors can contain gene sequences of different organisms, leading to the detection of multiple pathogens using a control vector or different genes of one organism to increase the accuracy of detection [37-42]. Therefore, these synthetic tools can be designed and used in molecular methods such as PCR-based methods, and isothermal amplification methods (LAMP and NASBA) [46]. The significant point in this issue is the primer properties because each molecular method requires specific primer features. For example, the primer properties in PCR-based methods and isothermal methods are different from each other at some points [47]. In a study, Menard *et al*. designed a synthetic plasmid construct for quantification of *Lactobacillus* spp, *G. vaginalis*, *etc*., by qPCR method [48]. Also, synthetic constructs are used for multiplex PCR to detect pathogens. In this field, Pourmahdi and colleagues designed a hybrid chimeric vector for the simultaneous detection of *Francisella tularensis* and *Yersinia pestis* using a multiplex PCR reaction (Fig. 1A). In this work a molecular diagnostic assay was developed, with design a new plasmid construct containing the conserved *caf1* (F1 capsule antigen) and *fopA* genes from *Y. pestis* and *F. tularensis*, respectively that has used as positive control [1]. In 2004, Charrel studied pathogens that are potentially dangerous for human health. They designed positive control plasmids containing the sequence of pathogens for detection. The map of the designed plasmid by them is shown in Fig. 1B [38]. In 2020, Samiminemati and co-workers design and construct a positive control contains a specific region of the *com1* gene for the diagnosis of *Coxiella burnetii* and the sensitivity of the reaction was determined about 2.1×10^{-5} ng μl^{-1} of DNA [33].

In 2013, Caasi and colleagues designed a PCR positive control for *Barley* yellow dwarf virus, *Soilborne* wheat mosaic virus, *Triticum* mosaic virus, and *Wheat* streak mosaic virus [40]. These constructs can also be used in quantitative PCR. In a related study, Janse used a synthetic construct as an internal control of amplification in a real-time PCR reaction. They designed and constructed an amplification control for molecular diagnosis of *Bacillus anthracis*, *Francisella tularensis,* and *Yersinia pestis* using multiplex qPCR [49]. In 2009, Carrera and Sagripanti engineered an artificial plasmid for simulation and detection of multiple biological threat agents (Fig. 2). For this, a non-virulent construct from 10 to 12 viral and bacterial agents was designed in order to develop detection and identification in molecular assay [7,39].

Also, in the LAMP or NASBA, which are isothermal methods, the primers have some particular features, which should be considered while designing constructs and primers. For example, during the LAMP method, three sets of specific primers are necessary to perform the amplification. This method requires specific enzyme and

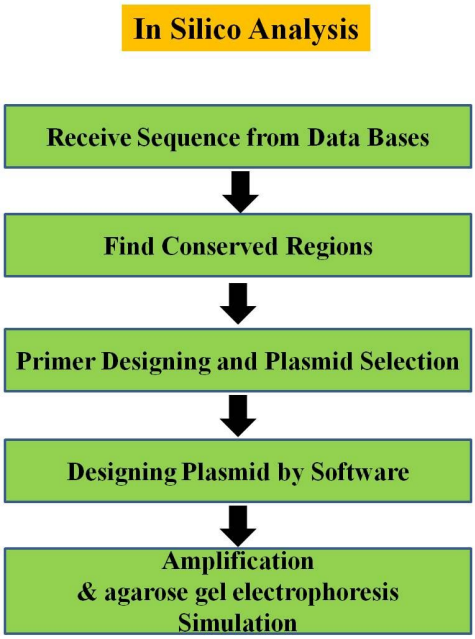

Fig. 2. Schematic presentation of a chimerical construct. (A) The genomic sequence of each agent has represented with yellow segments. (B) The organization of specific sequences according to 10 viral or bacterial agents sequences.

target sequences to run the reaction. Therefore, in these cases, it is necessary for the related sequence of the pathogen in the control plasmid to be long enough for amplification. These vectors can be designed for this aim to be used as a similar sequence to develop several isothermal amplification techniques for targets with individual features [50-52].

Bioinformatics Tools for Designing Synthetic Constructs

As mentioned before, particular data about the genomic regions and gene sequences are required to design a synthetic construct. The main databases for the organisms' genomic information are the National Center for Biotechnology Information (NCBI) Gene bank [53], DNA Data Bank of Japan (DDBJ) [54], the European Molecular Biology Laboratory (EMBL) [55], and European Bioinformatics Institute (EMBL-EBI) [56]. Searching for the names of organisms in databases gives us information about them such as complete or partial genome sequences, genome mapping, and further similar data. These databases report gene sequences stored at Gene Bank, which is used to design a hybrid vector. In the beginning, we should study the organism and obtain information about the specific genes to recognize via this approach. For this purpose, the name of the organism is searched as a query in a database like NCBI to get information about the organism and its genomic regions or specific genes that can be used as the target for the molecular diagnosis. In the following step, the obtained sequences from data banks should be analyzed and compared to choose a region as the target gene. In this step, multiple alignment tools can be used like Molecular Evolutionary Genetics Analysis (MEGA BLAST) [57], multiple sequence alignments (Clustal Omega) [58], or similar offline and online software (Fig. 3). These software help us to align the sequences to achieve protected regions during evolution called conserved regions [59]. These conserved regions are utilized as the target sequence for designing and making the hybrid vectors. The next step is primer designing for the target sequence according to the target of the vectors used in the diagnostic tools. Based on the type of the diagnostic method, primers design is carried

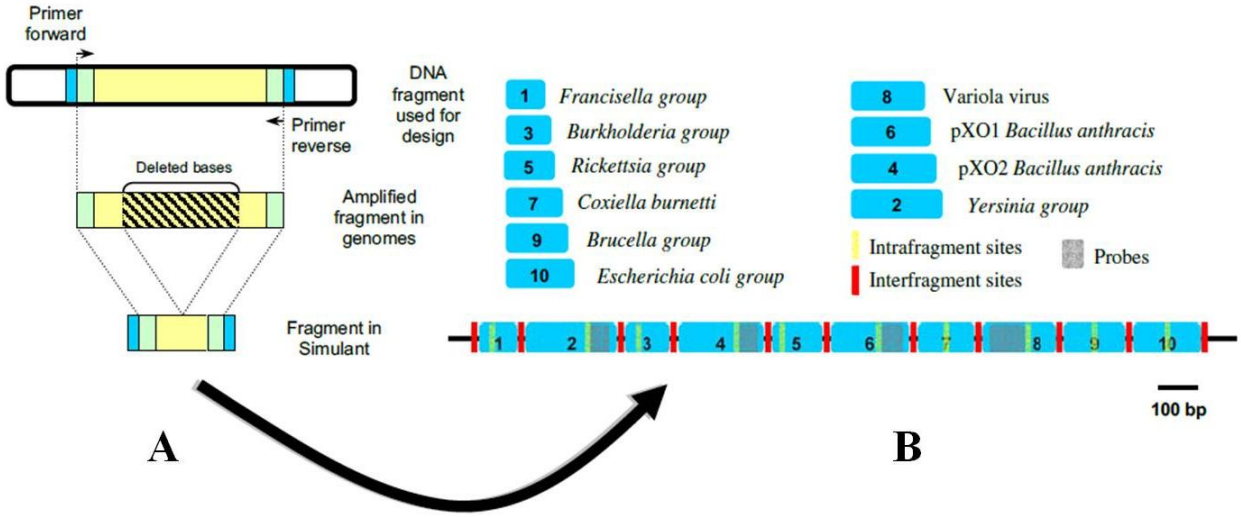

Fig. 3. Schematic of step by step of the chimerical vector designing.

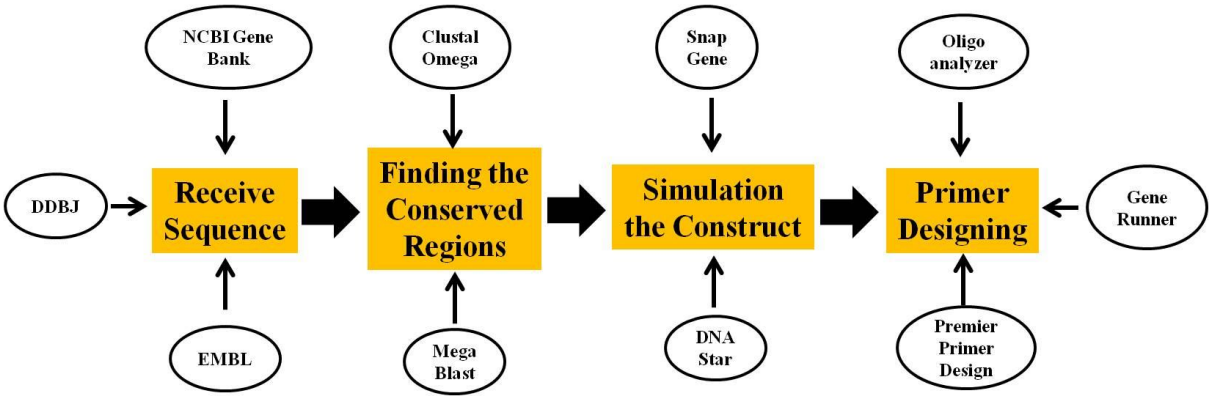

Fig. 4. Required databases and software.

out with different features using various software. Some special software include Oligo analyzer [60], Oligo tools of Gene Runner [61], Premier primer designs [62], *etc.* In the following, the designed primers should be considered and blasted using NCBI primer blast [63] to ensure the specificity of primers and amplicon sizes (Fig. 4).

The final step for designing positive control is to add some sequences to the primary sequence in order to create remarkable features. For this, restriction enzyme recognition sites will be added to the 5′- and 3′- end of the sequence for cloning into a plasmid or multiple digestion sequences from each other. Another variation in the sequence is to make a difference in the amplifiable sequence of the construct relative to the original organism. Utilizing this idea may cause an extreme decrease in false-positive results. Finally, the engineered sequence is cloned into the design plasmid and then it is transformed into the bacteria (host) for amplifying and banking.

There are a lot of applications to simulate cloning the fragments into plasmids. The SnapGene (GSL Biotech; available at snapgene.com) is a common offline software in this field. This software has many tools for simulating a lot

of molecular biology analyses such as PCR, restriction enzyme recognition sites on the sequence, restriction cloning, agarose gel electrophoresis, etc. Using this software gives enough information about the sequence, vectors, primers, and also the final map of the vector after cloning the fragment [64]. DNASTAR Lasergene is another offline software that is widely used for simulation of cloning and other similar subjects (Fig. 4). It includes Molecular Biology, Protein, and Genomics to provide our requirements at this point for each simulation study [65].

Application of Positive Controls in Diagnostic Kits

Currently, many companies produce specific diagnostics that differ in terms of diagnostic principles and materials used. In the serological kits, the ELISA method is more common than others because it is easier and cheaper. The IFA method is more sensitive and specific than the ELISA, but it is very expensive and requires specialized operators. Therefore, diagnosis of high-risk infectious agents using serological methods and kits has limitations. Also, the need for time for antibody reaction is another disadvantage of these kits [66]. These limitations necessitate the creation and use of molecular methods in the diagnosis kits. There are many diagnostic kits based on molecular approaches such as multiplex PCR, quantitative PCR, LAMP and NASBA. In this filed, the multiplex PCR and real-time PCR methods are more common because of their sensitivity and specificity. Also, these methods are usually preferred by companies because of using thermocycler devices and simple reactions for the laboratory staff. The use of synthetic constructs as a positive control in diagnostic kits is a proposed method to prevent contamination, as well as the safe detection of microorganisms. Accordingly, the Qiagen company has produced the microbial DNA positive control (10X) that is a pool of synthetic DNA templates for ensure the functionality of each microbial DNA qPCR assay using serving as targets for each microbial DNA qPCR assay.

CONCLUSIONS

The identification of microorganisms and pathogens is critical to prevent diseases. Additionally, accurate detection of the pathogen is a really important issue in bacterial, viral, and fungal contaminations. Therefore, immediate detection may cause a lower spread or decrease the associated damages. According to this, several diagnosis methods are used and various results have been reported. The oldest method in this field is culturing the bacteria to identify by visual colony characteristics. After evaluation of *in vitro* gene amplification using PCR technique and the manufacture of thermocycler device, molecular biology and molecular diagnostic assays improved, extremely. In the following, molecular methods were applied widely for the identification and detection of microorganisms. These methods are more accurate and more reliable than the previous methods because they detect specific areas in the genomic region. However, molecular approaches are usually preferred for detection but there are some limitations to this issue. These methods amplify the genomic regions so the genomic material or the native microorganism is necessary to perform the reaction. Nevertheless, it is impossible to access the rarely-found microorganisms in some countries. One smart solution way is using the synthetic or chimeric vectors which contain conserved genomic regions of the microbes. Chimerical hybrid vectors can be designed using various software in order to simulate the diagnosis mthods for each target organism. These vectors are usually synthesized artificially according to the genomic information in the associated databases. Applying synthetic vectors instead of the native pathogen is the most important utilization of these tools for the detection of high-risk infectious agents. These pathogens require special biosafety laboratory conditions and the probability of infection is possible for the laboratory staff. Consequently, using this approach is suggested to develop diagnostic assays, as well as the safe detection of microbes. For this, we recommend that the synthetic construct be used as an intelligent method to detect inaccessible or high-risk pathogens. To conclude, these vectors are capable to be used as a positive control for the development of the amplification assays.

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Conflict of Interests

The authors declare no conflict of interest.

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