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A review on molecular methods in the detection of foodborne pathogens

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ABSTRACT

Food safety is a major healthcare concern globally. Foodborne pathogens are held responsible for the elevated incidence of foodborne diseases worldwide, which also has an economic impact more so in developing countries. For these reasons, it is crucial to supply food which has undergone testing and is safe for consumption. Initially, testing for pathogens solely relied on culture-based methods; which turned out to be time-dependent and labour intensive. Identification of pathogens in a short time with standard accuracy stood as a challenge. After decades of research, rapid detection methods were developed to ensure food safety at an industrial level. Rapid techniques comprised of nucleic acid-based methods like PCR, multiplex PCR, nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP) and DNA microarray; immunological-based methods like enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassays and optical, electrochemical and mass-related biosensor were the popularly used methods. DNA markers were also commercially available for detection of target organism in the sample. The main purpose to devise these molecular techniques was to provide with rapid, highly sensitive and specific results while being economically convenient. This report summarizes the existing molecular methods and improvements that were made over the years for detection of foodborne pathogens.

Keywords: *Foodborne Pathogens, Food Safety, Molecular Methods for Detection, Conventional Methods, Culture Based Methods, Nucleic Acid Based Methods, Immunological Based Methods, Biosensors, Markers*

1. INTRODUCTION

In humans, microorganisms are a part of the gut and skin microflora, which are harmless and have proven to be important for good health. However, some organisms like *Bacillus subtilis, Bacillus cereus, Salmonella* Enteritidis, *Staphylococcus aureus and Escherichia coli* O157:H7 are commonly seen to be the pathogenic microorganism responsible for causing foodborne diseases in humans and affecting the society at large. These harmful microorganisms can gain entry into the human body through the gastrointestinal tract via contaminated food and cause various diseases.

With the increasing demand for street food and ready-made, minimally processed food, public health becomes a serious concern. Pathogenic microorganism can be present in any of the food items available in the market, like fruits, vegetables, partially cooked meals and raw or undercooked meat, which may not be processed adequately. This might cause foodborne outbreak over a large population amongst which the young, elderly and pregnant women would be the worst affected as their immune system are compromised. This can be prevented by installing essential analysis techniques to check for microbial contamination of food to ensure the safety of the population and cut down the incidence of foodborne diseases.

Previously, conventional methods like culturing the sample on the media, was the preferred mode of detecting pathogen. This method was less expensive, simple and did not require a skilled team to perform the protocol. But as this was a very time-consuming process, hence was necessary to be replaced by rapid techniques. Rapid techniques can be classified into nucleic acid-based techniques, immunological techniques and use of biosensors, which speeded up the process and provided sensitivity to some extent. This report represents the gradual shift from conventional methods to rapid molecular methods and later on discusses the need to further innovate advanced methods to overcome the limitations of the molecular methods as well.

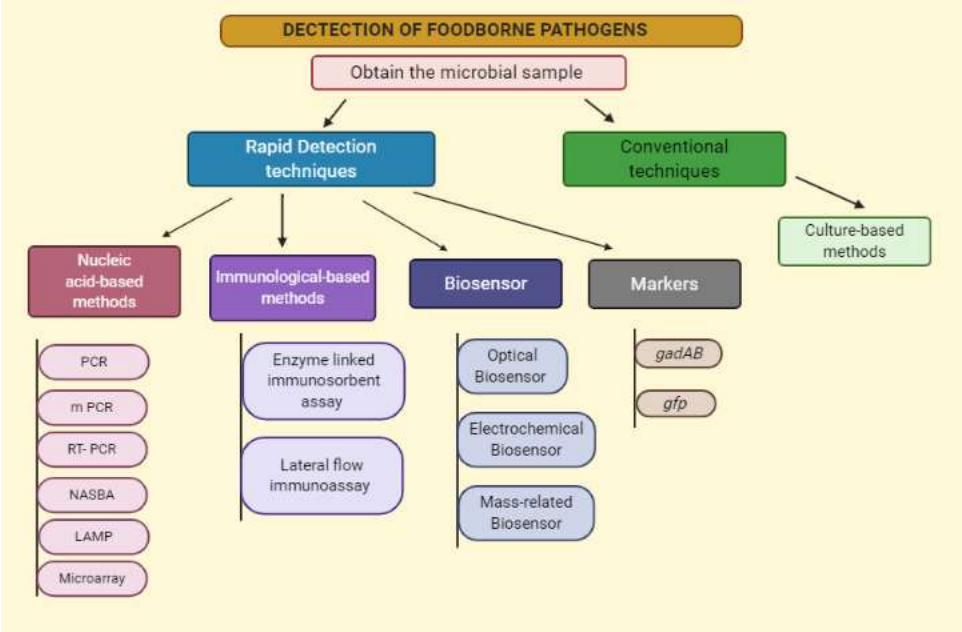

Fig. 1: Mapping of detection of foodborne pathogens

2. CONVENTIONAL METHOD

2.1 Culture-based methods

They are the oldest methods of detection for pathogens therefore regarded as a traditional technique. This technique simply involves the plating of the food sample on a suitable culture media for the enumeration of pathogens. For identification of pathogen, sample enrichment step is required followed by various other biochemical and serological confirmatory testing. A total of 2-3 days is required for the culture results to develop and another 7-10 days for the confirmatory testing results. This is one of the biggest shortcomings for any pathogen analysis technique, as there is an excessive time lapse with can result in fatality. Although this technique is considered as the gold standard, it is time-consuming and laborious. For this reason, it makes culture-based technique unsuitable to be used on a larger scale in food industries.

Strains of *E. coli* O157:H7 was seen to be growing on Sorbitol MacConkey agar (SMAC) media by which its presence can be identified. In fact, bacterial species differentiation can be made between *Yersinia enterocolitica* and other bacteria, indicated by purple/blue colonies on selective Cefsulodin-Irgasan-Novobiocin (CIN) media.

Many microorganisms become metabolically inactive as they encounter stressful environment. These microorganisms will return to normalcy but cannot be cultured in conventional media and facilitate pathogenic pathways. Such microorganisms are called viable but non culturable (VBNC). Detection of VBNC pathogens become very difficult and can cause serious problem to maintain food safety. Fluorescein isothiocyanate and acridine orange dye can detect VBNC pathogens as violet or blue and orange color respectively. Culture-based techniques can be combined with other methods to yield more promising results.

3. MOLECULAR METHODS

3.1 Nucleic acid-based methods

Nucleic acid-based methods become the most widely used assay and a powerful tool for the detection of foodborne pathogens as technology progressed through the years. This technique utilizes nucleic acids (DNA or RNA) sequences as a basis for detection. This can be performed by the hybridization of a synthetic oligonucleotide primer complementary to a specific nucleotide sequence. Many toxic substances are secreted by bacterial pathogens like *Clostridium botulinum, Staphylococcus aureus, Campylobactor jejuni* and *Escherichia coli* O157. These toxins are encoded by genes present in the genome of these pathogens and these toxin-related genes can be targeted by the nucleic acid-based methods for diagnosis of foodborne pathogens. Nucleic acid-based methods are carried out by simple polymerase chain reaction (PCR). PCR techniques has undergone crucial modification for much faster detection by developing multiplex polymerase chain reaction (mPCR), reverse transcriptase-PCR (RT-PCT), nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification.

[A] Polymerase Chain Reaction:

Kary Mullis's discovery of the PCR about 3 decades ago revolutionized the field of recombinant DNA technology. The basic principle of PCR revolves around the amplification of the target nucleotide sequence in cycles of the steps. Initially, double-stranded DNA is exposed to high temperatures for it to denature into single-stranded DNA. Then, two sequence-specific primers (reverse and forward) are synthesized which can anneal to the DNA template. As the primer bind to the complementary site on the DNA template, the primers are extended with the help of thermostable *Taq* DNA polymerase in the presences of ddNTPs. The PCR amplified products are identified by agarose gel electrophoresis and visualized under UV light as it is stained with ethidium bromide.

PCR technique can be used in the detection of pathogens by developing a specific primer complementary to the gene specific to the microorganism. *Listeria monocytogenes, Staphylococcus aureus, Campylobactor jejuni* and *Escherichia coli* O157 are few of the many foodborne pathogens which can be traced by PCR. Specific gene like the *fimA* gene of *Salmonella* and the *afa* gene of *E. coli* can effectively isolated the presence of these pathogens in the sample under study. As PCR is a more rapid, sensitive and a less

tedious technique, it is most preferred amongst the other detection techniques. Although this technique has shown some promising results, there are a few limitations like the inability to distinguish between live and dead cells. False positive results were generated due to non-specific binding of the primer leading to the assumption that PCR is a less reliable approach. Due to these shortcomings, the PCR technique was modified.

[B] Multiplex PCR (mPCR):

Multiplex PCR technique is much faster when compare to conventional PCR in terms of detection for the simple reason that mPCR uses multiple sets of primers. mPCR and simple PCR are similar in the basic principle, wherein mPCR has the advantage to simultaneous amplify multiple target sequence in one reaction system. Primer designing holds main significance in this technique to ensure proper amplification. While designing a primer, it is essential to keep in mind that the annealing temperature of the primers must be similar to avoid interference in the amplification process. Also, the concentration of the primer in the reaction must be maintained so as to prevent annealing between the primers to form primer dimers. Initially, only a few pathogens could be detected by mPCR but with certain advancement, around five pathogens could be detected at the same time.

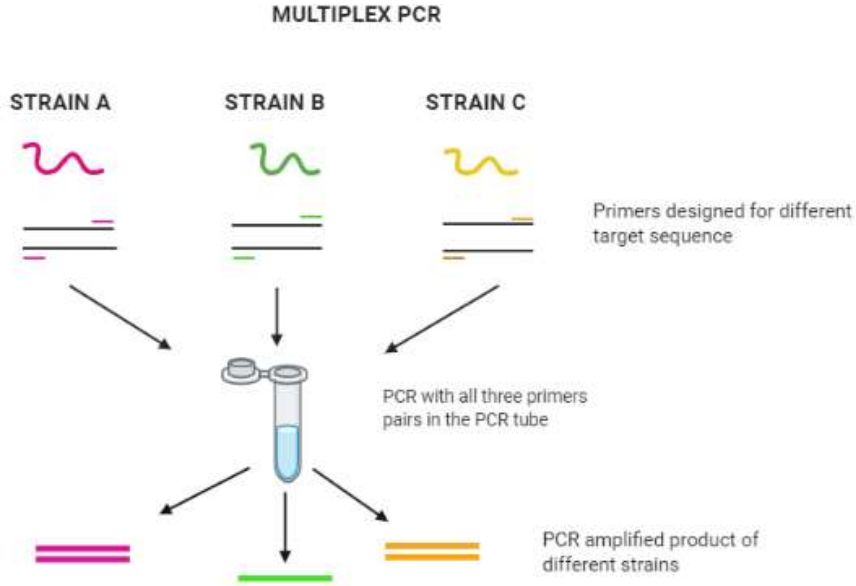

Fig. 2: Schematic representation of Multiplex PCR protocol

[C] Reverse Transcriptase-PCR (RT-PCT)

Reverse transcriptase-PCR is a version of conversional PCR which uses mRNA (messenger RNA) as the template. Using reverse transcriptase enzyme, the complementary DNA (cDNA) was synthesied for the mRNA of pathogen which would provide an evidence of metabolically active cells. The cDNA serves as a template for exponential amplification of the mRNA of the pathogen. RT-PCR can also be used to study the expression of the genes which are responsible for infection and growth. This technique can undertake rapid detection of foodborne pathogens. By using RT-PCR, *Salmonella* sp. could be studied and detected in pork and sausage food samples and mRNA of pathogenic *S.enterica* was studied for detection.

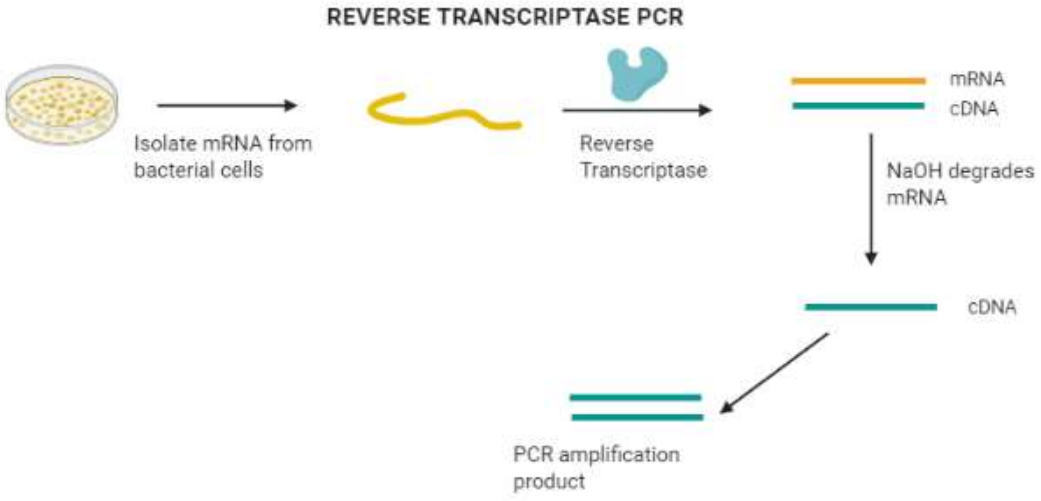

Fig. 3: Schematic representation of Reverse Transcriptase PCR protocol

[D] Nucleic Acid Sequence-Based Amplification:

Compton developed the nucleic acid sequence-based amplification (NASBA), which performs amplification of nucleic acids in isothermal conditions, which eliminates the requirement of thermal cycler equipment. This transcription-based amplification uses primers to recognize specific target sequence on RNA. This amplification process comprises of three steps. Firstly, a primer anneals to the single-stranded RNA and converts it to a cDNA with the help of avian myeloblastosis virus (AMV) reverse transcriptase. The template RNA is then digested by RNaseH, a second primer complementary to cDNA binds to it and is extended by reverse

transcriptase to produce double-stranded cDNA. Lastly, RNA transcripts are produces by the amplification process using T7 RNA polymerase. In each cycle of NASBA reaction, 10-100 copies of RNA molecules were formed. The detection sensitivity range for this technique was established to be 1cfu/mL. The NASBA products were viewed on agarose gel or enzyme gel assay which were laborious and not economical. Thus, improvements were made to NASBA which used fluorescently tagged probes, making detection for feasible. The improved NASBA was called Real-time NASBA which had the ability to differentiate between viable and non-viable cells as the sample was treated with RNase which allowed the degradation of target mRNA in the dead cells. This technique is a promising diagnostic tool in the detection of microorganisms like *Campylobactor jejuni*, *Campylobactor coli* and *Listeria monocytogenes* in various food

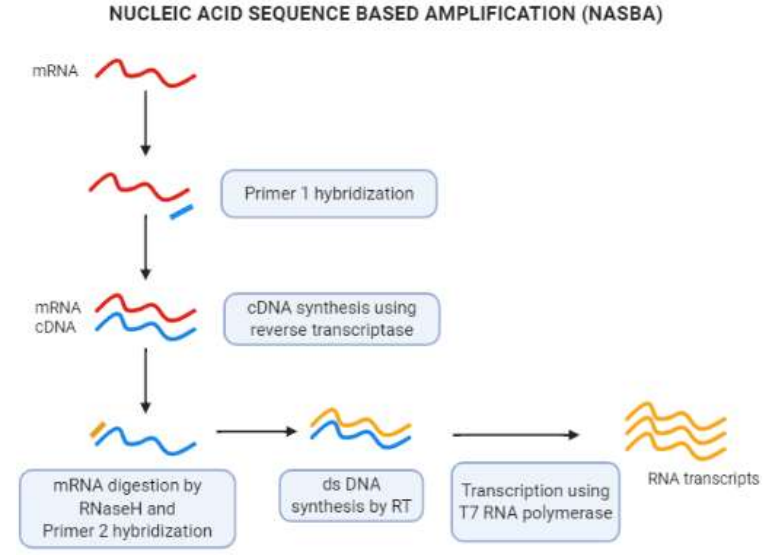

Fig. 4: Schematic representation of NASBA protocol

[E] Loop-Mediated Isothermal Amplification (LAMP):

Loop-Mediated Isothermal Amplification, a novel nucleic acid amplification process developed by Notomi et al in 2000, allows rapid, sensitive and cost-efficient method for detection of target sequence in the food sample. It is a single-step amplification process based on the auto-cycling strand displacement principle, conducted isothermal conditions ranging between 59° C to 65° C. LAMP protocol involves the use of *Bacillus strearothermophylus (Bst)* DNA polymerase of the strand displacement step, and employ four primers, two outer primer and two inner primer, lining six specific target DNA sequences. The procedure can produce 10,000 folds larger amount of amplicon less than 60 minutes, more so as there is no need of DNA purification.

For the detection of food pathogens, LAMP was used to track the *stx*A_2 gene in *Escherichia coli* O157:H7. Other microorganism like *Salmonella, Listeria and Campylobacter were* subjected to LAMP for detection. Variants of LAMP were developed, like reverse transcriptase LAMP, real time LAMP, *in situ* LAMP and multiplex LAMP.

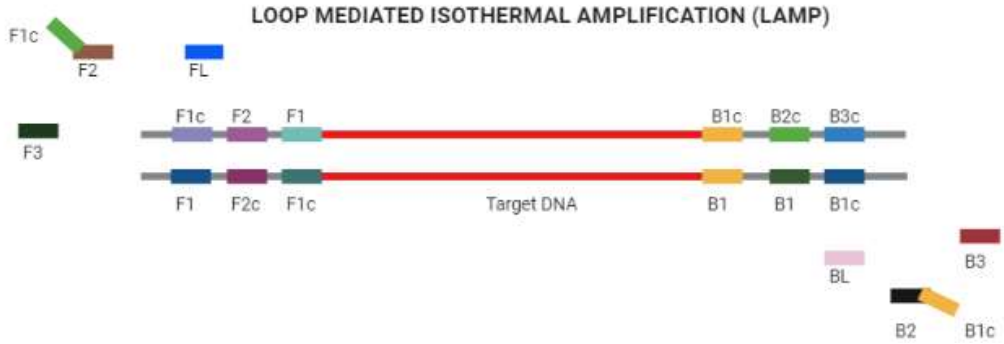

Fig. 5: Overview of Loop mediate isothermal amplification (LAMP) with primer sets. F3: forward outer primer, FL: F-loop primer; B3: backward outer primer, BL: b-loop primer.

[F] Microarray:

DNA microarray, more commonly called as DNA chip or biochip, is a small, 2D matrix which has a collection of microscopic DNA spots printed on glass or silica solid support. An amount of few picomoles (10^{-12} moles) of DNA sequence are compressed into each spot which serve as a probe for hybridizing to a target. The probe-target hybridization can be identified by using fluorophores or chemiluminescent-labeled probe which can be further detected by computerized software. As thousands of probes are present on the slide, several microarray detections can be performed simultaneously, therefore drastically accelerating the efficiency. The only challenge that remains is to ensure microbial safety in the food industry for the detection of minute traces of pathogenic microorganism in a large food sample, which might also involve background organisms to interfere with the matrix. Therefore, it is necessary to utilize PCR for amplification to increase sensitivity of the testing. DNA microarray technology has shown successful detection results in waterborne pathogens with pose a threat to humans on consumption of seafood. In case of foodborne pathogen,

microarrays were designed to track internal transcribed spacer (ITS). By using this method, *L. monocytogenes, S. aureus, E. coli* and a few more pathogens were detected.

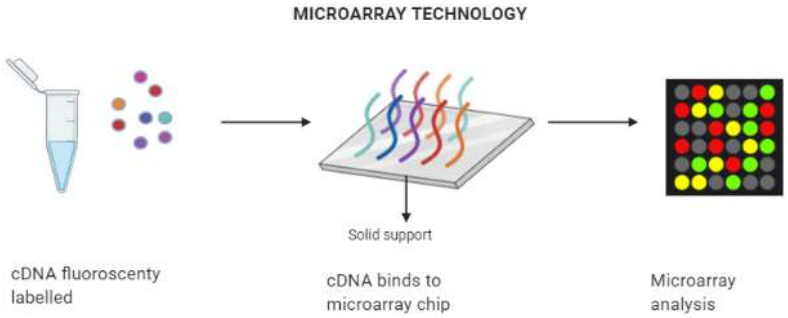

Fig. 6: Schematic representation of Microarray technology

3.2 Immunological-based methods

Immunological means for detection of pathogens is based on antigen-antibody reactions. Specific antibodies are produced in animal models as a response to induced pathogenic attack. The pathogens secrete toxins or antigens which are recognized by the specific antibody. The strength of antigen-antibody binding determines the sensitivity and versatility of this detection technique. With the development in hybridoma technology, monoclonal antibodies and polyclonal antibodies are involved in immunological-based methods of detection of pathogens.

[A] Enzyme-linked immunosorbent assay:

Enzyme-linked immunosorbent assay is one of the fastest and most commonly used immunological methods to detect pathogens in food. Sandwich ELISA is the most common variant of ELISA used which employs two antibodies. The primary antibody is immobilized on the microtiter wells, to which target antigen in the form of bacterial cell of toxics from the food sample, are exposed. The antigen binds to the antibody on the microtiter well and the unbound antigens are washed away. This is followed by the introduction of a second antibody which is tagged with an enzyme (like horseradish peroxidase, alkaline phosphatase or beta-galactosidase) that goes and binds to the antigen. The antigen is "sandwiched" in between the two antibodies, hence the name of this technique. Later on, the presence of this sandwiched complex is detected by adding a color-less chromogenic agent which is degrades by the enzyme to give a colored product. *Clostridium perfringens*, *Vibrio parahaemolyticus* are a few pathogens found in seafood which can be detected by sandwich ELISA. ELISA offers accuracy at the cost of specialized equipment and skilled technician for carrying out the process. Therefore, a more rapid, easy, cost efficient and dependable technique was required.

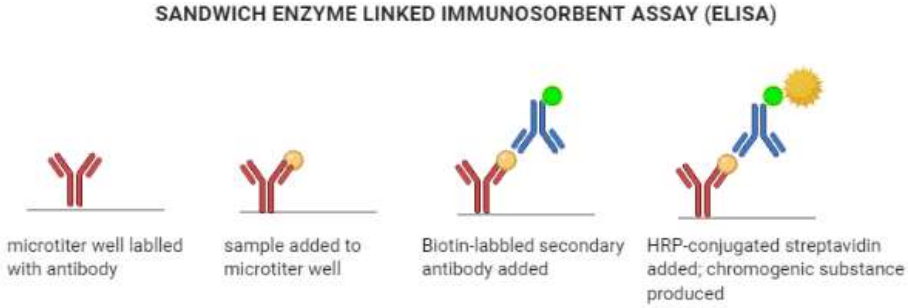

Fig. 7: Diagrammatic representation of Sandwich ELISA

[B] Lateral flow immunoassays:

Lateral flow immunoassays were developed to conduct a most practical and on-site detection. The lateral flow immunoassay chip consists of four sections, the sample pad, conjugate pad, followed by the nitrocellulose membrane and finally the absorbent pad. The sample is put on the sample pad which migrates through the section via capillary action. As the sample enters the conjugate pad, it interacts with the antigen or antibody (labeled with a chromogenic substance) present, forms a conjugate. As this conjugate pass through the lines in the nitrocellulose membrane, it is immobilized by an antigen or antibody here. The color appears two to ten minutes after the sample is introduced. *E. coli* O157 and *Salmonella* are detectable in the food sample by this method.

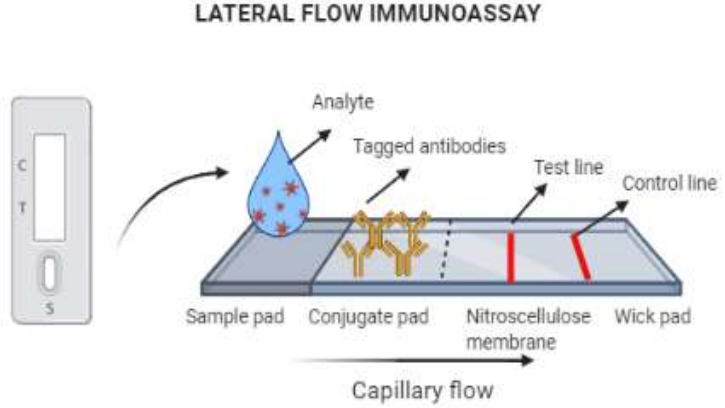

Fig. 8: Schematic representation of Lateral flow immunoassay

3.2 Biosensor

Biosensor is an analytic tool which converts a biological response into an electrical signal via two components: a bioreceptor, responsible for the recognition of the target analyte, and a transducer, that converts this bio-recognition energy into electrical signal by using the means of optical, electrochemical or mass-related techniques. Unlike the methods discussed above, biosensors do not require a sample enrichment step and is very easy-to-operate. In the recent years, the use of biosensor in the field of pathogen detection in food industry as shot up tremendously. It the preferred method for detection compared to other standard methods due to its real time response. Several varieties of biosensors are available in the market based on the difference in their bioreceptor and transducer- optical, electrochemical and mass-based biosensors to name a few.

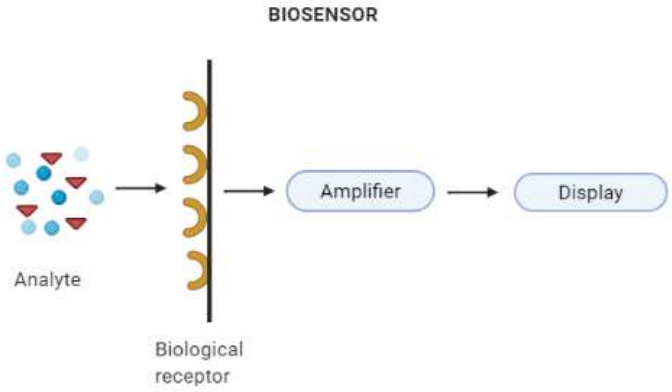

Fig. 9: General representation of Biosensor

[A] Optical biosensor:

Optical biosensors are a very reliable tool for the detection of pathogens in food substance and the most commonly principle used is surface plasmon resonance (SPR). SPR owes its level of sensitivity to reflectance spectroscopy. In this method, the biosensors are immobilized on a metal surface, emitting electromagnetic radiations of a particular wavelength which creates a strong resonance with the electron on the metal surface. If pathogen is present in the food sample, it binds to the metal surface, changes the refractive index and leads to an alteration in the electron resonance. Just like SPR uses resonance, there are other technological tools which can be used for detection by change in amplitude, frequency or polarity of light. SPREETA biosensor and BIACORE 3000 are the commercially available optical biosensors which has detected *E. coli* O157:H7 and *Listeria monocytogenes* and *Salmonella* respectively.

Optical biosensors are the most used biosensor due to its sensitivity, specificity and compact design. As it is a challenge to maintain stability of the immobilized biocomponents, biosensor can be marketed better if improvements are made in aspects of quality assurance, cost efficiency and calibration issues.

[B] Electrochemical Biosensor:

Electrochemical biosensor is another type of biosensor which is not a preferred technique. Although this biosensor can handle large amounts of food sample at once, electrochemical biosensors have low sensitivity range as the food matrices might interfere with the analysis. This will require multiple washing step which makes it unsuitable for detection of microorganisms present in low concentrations. Despite the drawbacks, *Bacillus cereus* was identified in strawberries and tomatoes samples with a detection range of 35-88 CFU/mL.

[C] Mass-based Biosensor:

Mass-based biosensors are mass- sensitive technique which detects the slightest change in mass. This method employs piezoelectric crystal which vibrates at a particular frequency as electric impulse is induced. The bioreceptors (i.e., antibodies) are immobilized on the crystal onto which the pathogen may bind. As the target antigen (in this case pathogen) bind to the crystal, the frequency of vibration is considerably altered which in correlation to the added mass onto the crystal. Mass-based biosensor is the least preferred amongst all the biosensors due to low sensitivity and longer incubation period; yet *E. coli, Salmonella and Listeria monocytogenes* which detection limits of 10^9 CFU/mL, 1 x 10^5 cells/mL and 1 x 10^7 cells/mL.

3.4 Markers:

Traditional food pathogen detection techniques like culture-based methods are very time-taxing and labor intensive whereas immunological and nucleic acid-based methods need large samples and do not produce on-site results. Novel biological elements like bacteriophages have been studied to be used as a probe for pathogen detection as this entity has high specificity towards the host organism. Studies have shown that *gadAB* gene can be used as a marker for *E. coli* strains. It was understood that *gadAB* marker can be used to screen *E. coil* in a sample. These markers were called DNA probes as it comprises of genes. Green fluorescent protein (GFP) is used as a protein probe acquired from jellyfish *Aequorea victoria,* encoded by *gfp* gene. The *gfp* gene induced in the transformed strain of *E. coli* O157:H7 was proven to be useful in detection of pathogens.

4. CONCLUSION

For appropriate detection method must suffice the following requirement – high specificity (to detect on the target pathogen), high sensitivity (ability to detect traces of pathogen in a large sample), short time frame for results (within minutes or hours of time), operational and design simplicity (to avoid the use of prolonged protocols and specialized equipment) and economical. These

parameters ensure appropriate controls to maintain food safety. Tremendous growth has taken place in the field of food microbiology over the last twenty years, which has paved the way for the development of many progressive techniques.

Despite conventional culture-based being regarded as the "Gold standard" for its simplicity and cost effectiveness, it is a highly laborious and time-consuming. These limitations would be overcome by the formulating rapid detection methods. Therefore, this gave way for molecular methods to gain importance. Nucleic acid-based methods like PCR, mPCR, RT-PCR and DNA microarray provide high sensitivity and accuracy but require trained assistance and costly equipment. Alternatively, other nucleic acid-based methods like LAMP and NASBA are not only sensitive and specific but also cost-efficient as it does not need costly equipment like the thermal cycler.

Immunological-based methods included ELISA and lateral flow immunoassay which has strong sensitivity towards detection of pathogens and their toxins. Unfortunately, this method too has a limitation due to the presence of molecules in the sample that interfere with the analysis.

Furthermore, biosensor-based methods were gaining attention due to their rapidness in producing on-site results, no requirement for skilled personnel, at the same time being cost efficient. But to provide these benefits, biosensor-based methods compromised on sensitivity. Markers were developed to target a specific sequence in the food sample to detection the presence of pathogen.

In this report, the molecular methods are cumulative represented and their advantage and limitations discussed. Improvements in these techniques have also been mentioned. It is important to be noted that, detecting and isolating a foodborne pathogen in a single step is not possible. This needs to be carried out in every step of the detection process. The gap between performing the procedure and obtaining the results are expected to be reduced by the future advancements in this hot field of research. The need of real-time results could be majorly overcome by employing "flash" enrichment step. To compensate for these limitations, involvement of techniques like mass spectrometry, immunomagnetic separation and optical scanning are adapted. Therefore, this requires coordination of scientists from various disciplines of science would lead to a dramatic boost in the detection technologies to innovate methodologies to detect next generation pathogens and toxins.

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[12] All creative illustrations were made using web-based tools.

