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Review Article

Expanding the Frontiers of Bacterial Diagnosis through Bacteriophage Biotechnology

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ABSTRACT

In a bid to achieve microbial diagnostic precision and reduce diagnostic turn-around time, the development of technologically advanced novel techniques has been on the rise. There is a gradual phasing out of traditional diagnostic methods by more specific and highly sensitive molecular techniques. Asides from being technically demanding and cost-ineffective, these molecular methods have themselves not fulfilled perhaps the most essential diagnostic criterion of distinguishing between viable and dead bacterial cells. The use of bacteriophages as biorecognition elements for bacterial detectors offers numerous advantages in terms of cost, ease of accessibility, and high specificity binding of bacteriophages to their bacterial host. Biotechnological advancements further give bacteriophage use the leading edge as genetic modification of bacteriophage genome through the fluorescent gene insertion produces reporter bacteriophages. These recombinants can produce detectable fluorescent signals through intracellular lytic action, strictly in metabolically active bacteria cells. Fluorescent labelled enzyme-active and cell wall binding domains of bacteriophages also offer better alternatives to the use of antibodies as diagnostic markers because they are resistant to pH and temperature sensitivities. Overall, bacteriophage-based detection systems are less prone to detection errors and significantly reduce diagnostic time while also attaining high test sensitivity.

***Keyword*:** Reporter bacteriophage, Fluorescent protein, Biotechnology, Phage-based sensors.

Introduction

Microbial diagnosis forms an integral part of clinical, food, industrial, and environmental settings. This is consequent to the economic importance of bacterial infection and contamination in these fields. The course of the years has seen relentless strides in efforts geared towards the improvement of microbial diagnostic precision and reduction of turn-around time, through the development of novel diagnostic approaches that are capable of achieving adequate specificity and sensitivity even within complex samples.[1] Early advances in synthetic biology paved the way for the use of single-celled organisms as bio-recognition elements for detection biosensors. These organisms were embellished with signal-processing circuitry components, which could convert biochemical signals to readable electronic outputs.[2] These hybridized diagnostic systems showed early signs of remarkable advantages over the abiotic sensors based on nucleic acid hybridization or purified antibodies in that they were able to produce high bio-catalytic activity for signal generation.[3] These novel systems, however, fell short of expectation in sensitivity levels, as they were largely plagued by the effect of interference associated with culturing conditions.[4]

Biotechnological knowledge improved greatly in the subsequent years, thus, allowing the use of genetic modification techniques in altering the genetic composition of organisms of interest. These biotechnological advances proved extremely beneficial to the use of bacteriophages in developing more purpose-specific sensors, with improved signal output strength.[5] Directed evolution allowed for the engineering of recombinant bacteriophages, which can detect bacterial presence within minutes of contamination by bacteria, making it practicable to develop novel, near real-time microbial diagnostic systems.[6] As glaringly promising as the prospects of bacteriophage use in bacterial diagnosis look, coupled with numerous experimental evidence to substantiate its use in the development of novel technologically advanced diagnostic systems, it is, however, surprising that only a handful of commercially available phage-based diagnostic devices are clinically approved for diagnosis.[7]

Traditional Diagnostic Techniques

Conventional diagnostic methods such as traditional culture plating and antibody-based assays form the mainstay of bacterial diagnostics in clinical settings. Culture plating in particular has remained the gold standard for bacterial detection and antibiotics resistance profiling, as it comes with the advantage of a relatively low cost and relatively low technical demand.[8] It is associated with aseptic sample collection, serial dilution techniques, the use of selective growth media for different bacterial species, and a long period of controlled incubation conditions, which range from 10 to 24 hours or even more, all in a bid to achieve selective bacterial amplification.[9] Serotyping, which is an example of a traditional antibody-based technique uses cell surface antigen-antibody interaction for bacterial detection and classification criterion for epidemiological studies. It is still very much considered a certified approach for bacterial sub-speciation.[10] Improved antibody-based assays such as Enzyme-Linked Immunosorbent Assay (ELISA), which show higher specificity and sensitivity have soon grown in significance, especially in the detection of uncultivable micro-organisms such as *Mycobacterium tuberculosis*. The versatility of ELISA kits is affirmed by the fact that the detection of many foodborne pathogens is solely characterized by the use of ELISA kits.[9]

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Overall, these detection techniques are often associated with critical enrichment steps on which diagnostic precision is dependent. It is therefore, safe to assert that these techniques are grossly insensitive without their enrichment steps.[11] Worthy of note is the fact that not all pathogenic organisms of interest can be grown on culture plates and so, only a handful of bacteria of interest can be successfully detected using conventional plating techniques.[12] Culturing techniques, in particular, are prone to the effect of environmental contaminants which affect diagnostic precision. The issue of culture non-selectivity is attributed to the inability of these techniques to appropriately differentiate pathogenic organisms from commensal organisms in a complex biological sample.[13] While antibody-based assays on the other hand are considered as better improvements on culture plating in terms of higher sensitivity, homogeneity, direct recognition ability, and reduced assay time, they are associated with numerous production challenges such as high production cost and batch-to-batch inconsistencies. Their use is further hindered by antibody sensitivity to enzymes and the inability to differentiate between viable and dead bacterial cells.[14]

Molecular Diagnostic Techniques

The use of molecular-based diagnostic techniques in bacterial detection was prompted by the need to improve diagnostic precision and sensitivity levels.[15] Molecular diagnostic methods allow for organism characterization, speciation, and antibiotic susceptibility profiling almost simultaneously. Non-culture based techniques such as real-time Polymerase Chain Reaction (PCR), hybridization-based detection assays, and direct ribosomal gene analysis have opened up new perspectives in the diagnosis of fastidious bacterial organisms and enabled their nucleic acid quantitation.[13] Real-time PCR employs nucleic acid amplification system, which allows for bacterial identification of as low as one pathogenic organism in a biological sample. The design of pathogen-specific primer allows for nucleic acid amplification in repeated cycles to generate a billion copies of nucleic acid of interest within a short time. The amplified products are then examined by the use of nucleic acid probes.[16] Overall, these diagnostic techniques have been proven to show higher specificity and sensitivity than culture-based techniques in pathogenic identification.[15] The routine use of these advanced approaches has, however, still not been fully achieved and maybe unachievable for the obvious limitations of the cost of primer design, infrastructural requirements, demand for technical know-how in process-handling, and result interpretation, and labor-intensity they often require. These approaches are not rid of their technical deficiencies. They fail to discriminate between living and dead cells, thus, increasing the propensity for false-positivity.[17] The result interpretation is often dependent on the expertise of the personnel handling the process. Microbial diagnosis using PCR is as good as the quality of the primers adopted; hence a failed primer design would often result in off-target hits and species misidentification.[18]

Bacteriophage Characteristics which makes them Suitable Biorecogntion Elements

Bacteriophages are natural predators of prokaryotic organisms (Bacteria and Archae) that employ the host cell machinery for their replication.[19] The unique ability of bacteriophages to exhibit binding specificity for only their target host makes them ideal candidates for use as biorecognition elements in bacterial detection. The earliest records of the adoption of bacteriophages as diagnostic tools date far back to the earliest years of bacteriophage exploration as antimicrobial therapeutic options. C. Kirchhelle reported that the "Phage Typing" technique was a fundamental diagnostic and epidemiological tool in the 1930s.[20] In this technique, the sensitivity pattern of bacterial strains to bacteriophage set is used as the diagnostic indicator. Bacteria culture plates are treated with phage lysates which result in the appearance of phage plaques, which are reported as phage types.[21] The technique differed only slightly from traditional culture-based assays as it depended largely on the bacterial host growth rate in the formation of phage plaques, which of course was time-consuming. It is however worthy of note that the technique is still very much relevant in epidemiological surveillance of bacterial outbreak and this is because it fulfills the epidemiological criteria of bacterial classification. This is evidenced by the fact that it is still deemed as the gold standard for the epidemiological surveillance of *Salmonella typhimurium*.[22]

Bacteriophage host binding is profoundly strong, multivalent, and specific. The enormous attributes of robustness, low reagent cost, and relative ease of accessibility due to the ubiquitous nature of bacteriophages make the design of novel bacteriophage-based detectors easily achievable.[23] They have been shown to have numerous advantages in rapid bacterial detection as they are resistant to the denaturation effects of harsh conditions posed by organic solvents and high temperatures. They are considered more sensitive than conventional diagnostic systems because they can distinguish between viable and dead bacterial cells, a critical diagnostic criterion in which traditional diagnostic techniques and molecular-based methods have fallen short.[24] Bacteriophages offer numerous design options for bacterial detection systems beyond use as recognition elements. They can be used as immobilized biosorbents on sensing layers such as the surface plasmon resonance technique, which measures real-time changes in resonant frequency to determine bacterial count.[25] Bacteriophages can also be used as capture elements for targeting bacteria in complex samples by binding them to micro or nanoparticles to form bioconjugates.[13] The design of bacteriophage-based detection systems is aided by the relative ease of modifying the bacteriophage genome to increase phage-bacteria complex identification through the incorporation of fluorescent proteins, affinity tags, and chemical labels.[26] A summary of the comparison among traditional diagnostic techniques, molecular methods, and bacteriophage based detection is presented in (Table 1).

Reporter Bacteriohage Technology

Bacteriophage bioengineering allows for the incorporation of fluorescent reporter genes into the bacteriophage genome, thus, allowing for the expression of fluorescent proteins within only metabolically active bacterial cells, which makes bacteria detection rapid and easy to achieve (Figure 1).

This was experimentally demonstrated by Loessner *et al.* using *Listeria* recombinant bacteriophage A511 in the detection of *Listeria monocytogenes*, a bacterial pathogen that has been identified as a culprit of food contamination.[6] Homologous recombination of *Vibrio harveyi* derived *luxA* and *luxB* reporter gene fusion (*luxAB*) into the major capsid protein gene of the Listeria bacteriophage resulted in the generation of detectable luminescence within 2 hours of phage application to *Listeria monocytogenes* contaminated food product at concentration as low as 5×10^2 *L.monocytogenes* cells/ml. In a more recent study conducted by Meile et al, nanoluciferase proteins (*Nluc*) were incorporated into the genomes of broad range phage A511 and two other serovar specific Listeria phages (A500 and A006). The broad range nanoluciferase based phage was able to detect 1 Colony Forming Unit (CFU) of *Listeria monocytogenes* in 25 g of artificially contaminated food products in less than 24 hours, while the modified serovar specific phages allowed for Listeria serovar differentiation, thus, providing substantial information on the estimation of bacterial isolate virulence, which can be useful in the epidemiological investigation.[27]

Mycobacteriophages have been successfully re-engineered to express reporter genes such as *ZsYellow* genes, green fluorescent protein (GFP) genes, and luciferase reporter genes in their non-essential regions in many experimental studies for *Mycobacterium* spp detection. One of such was undertaken by Piuri et al to demonstrate the use of GFP or *ZsYellow* fused TM4 bacteriophage to detect *Mycobacterium tuberculosis* through fluorescence detection by flow cytometry or by a fluorescent microscope.[17] The method was not only found useful in bacteria detection but also simultaneous antibiotic susceptibility testing for rifampicin or streptomycin-resistant strains through drug-induced fluorescence suppression in antibiotic susceptible cells. Drug sensitivity testing was achieved in enhanced biosafety conditions in less than 24 hours and this was possible because fluorescent proteins are resistant to paraformaldehyde treatment needed for sputum fixation. The results corresponded with that of an earlier work conducted for drug susceptibility testing in

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clinical *Mycobacterium tuberculosis* isolates, using luminometric detection of luciferase reporter phages.[28] The clinical isolates were pre-treated with isoniazid and rifampin antibiotics before administration of the reporter phages. Detectable photon emission caused by the presence of luciferin protein and cellular ATP from antibiotic susceptible cells was measured using a luminometer and a Bronx Box, both yielding 100% sensitivity to multi-drug resistance and a remarkable diagnostic turn-around time of 54 to 94 hours, compared to three weeks needed for agar proportion technique. A meta-analysis study on performance characteristics and diagnostic accuracy of FASTPlaqueTB assay, in-house phage amplification assay, and Luciferase Reporter Phages (LRPs) revealed 100% sensitivity and specificity of LRPs in the detection of rifampicin resistance in *Mycobacterium tuberculosis* isolates in 7 to 8 studies conducted.[29]

Reporter phage technology has been successfully employed in the detection of *E. coli* 0156:H7 strain through the use of recombinant bacteriophage PP01, which had a GFP- fusion tag inserted within its small outer capsid (SOC) protein. Bacteria detection was achievable within 1 to 3 hours using high multiplicity of infection and host cell membrane phage adsorption viewed under a fluorescent microscope.[30] A later study conducted by Tanji *et al* adopted recombinant lytic-deficient T4 bacteriophage as a substitute for PP01 bacteriophage due to the impact of lytic action on bacteriophage detection. The T4 bacteriophage which had an altered lysozyme gene was coupled with a GFP fusion tag in its SOC protein. This allowed the reduction of detection time to 10 to 30 minutes.[31] The recombinant phage was however not successful in the detection of all *E. coli* strains due to the lack of broad host infectivity range.

Fluorescent labeled enzyme-active and cell wall binding domains of bacteriophage endolysins have been suggested as alternatives to the use of antibodies as diagnostic markers because they are characterized by high specificity binding and host affinity. Antibodies on the other hand are prone to pH and temperature sensitivity, high aggregation propensity coupled with high cost and time consumption.[32] In a study conducted by Kretzer *et al.,* antibodies were replaced with cell wall binding molecules CBD118 and CBD500 derived from recombinant *Listeria* phage endolysin. These were expressed as fusion proteins with histidine-tagged GFP reporter genes on *E.coli* for the detection of *Listeria monocytogenes.*[33] The study reported that the paramagnetic beads coated with the endolysin derived CBD- molecules were able to immobilize and separate more than 90% of the *Listeria monocytogenes* cells within 20 to 40 minutes in both natural and artificially contaminated food samples. The sensitivity of the technique was highly remarkable, with a turn-around time far superior to that obtainable from established procedures. The assay exhibited host-specific binding unhindered by cross-reactivity reactions. In a similar study, cell wall binding domains of bacteriophage derived CPT 1L endolysin labeled with GFPs were tested against dairy-related *Clostridium* species. The GFP-CBDs were able to detect 17 of the 20 *Clostridium* spp tested as well as to detect clostridial spores.[18] In a more recent study conducted by Santos *et al.,* CBDs of PlyP123 lysin were fused with GFPs and successfully bounded to all strains of *Paenibacillus larvae* in ERIC genotype.[34]

Limitation of Reporter Bacteriophage Technology

The limitations of reporter bacteriophage technology are not exactly associated with the availability or unavailability of cutting edge bioengineering tools, but rather the natural properties of bacteriophages themselves. Many bacteriophages have narrow host infectivity ranges, which mean their use in diagnostics is often restricted to only a few bacterial strains of single species. While this accounts for higher specificity for bacteriophage-based detection systems, it implies reduced versatility of reporter bacteriophages in bacterial diagnosis.[35] The challenge of narrow host infectivity ranges has been circumvented in therapeutic use via the 'cocktailing' of bacteriophages with complementary host ranges. This approach can also be adopted in the use of reporter bacteriophages for bacterial detection. The complex particulate nature of bacteriophages, coupled with their large molecular weight has been identified as a major hindrance to the adoption of reporter bacteriophages as bio-recognition elements.[36] The large size of bacteriophages might create a distance between the reporter phage and the bacteria target, which in turn affects the fluorescent signal generated on lysis. The nature of phage lytic action does not allow prolonged analysis, especially in samples of low bacteria concentration. Generated fluorescent signals might be lost while the other bacteria cells are still being bound to the sensor.[36] In the use of reporter phages as immobilized capture elements on sensing layers, steric hindrance might be created by parts of the phage virion not involved in bacterial binding. This challenge can however be circumvented through the use of parts of bacteriophage such as the enzyme-active and the cell wall binding domains of bacteriophage endolysin as bio-recognition elements.[37, 38]

Future Prospects

Experimental evidence reveals the impact of recent advancements in molecular biology on rapid and precise modifications of the bacteriophage genome. These cutting-edge tools have made phage engineering, which was initially a complex and time-consuming task a rapid and easy to undertake process, even for less well-characterized bacteriophages.[39] Phage genome engineering offers the opportunity for the redesign of the phage genome to achieve specific needs. Such needs include the production of recombinant reporter phages with broader infectivity ranges through receptor binding protein or baseplate engineering.[35] This would in turn increase versatility of use while also reducing production cost for the design of phage-based biosensors for specific bacterial strains.

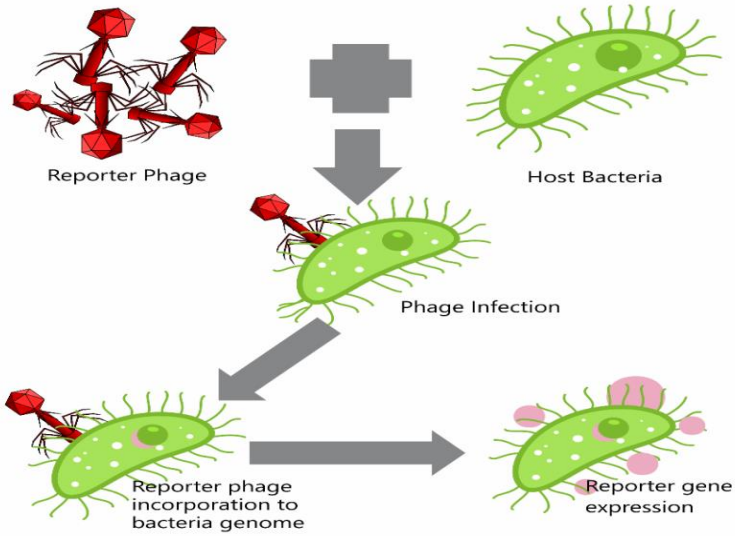

Figure 1: Reporter Bacteriophage Technology (Reporter phages use host bacterial cell replication machinery to express infused reporter genes as fluorescent proteins, which produce detectable bioluminescence)

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The engineering of recombinant bacteriophages which would be able to evade the numerous intracellular defense mechanisms of host bacteria could prove very beneficial to the use of reporter bacteriophages in bacterial detection. Methylation of certain portions of the bacteriophage genome could help reporter bacteriophages in successfully infecting bacteria cells with restriction-modification system.[40]

The engineering of anti-CRISPR proteins alongside fluorescent proteins in the phage genome could help reporter phages exert their lytic action even in the presence of the CRISPR-Cas system. With the numerous advancements being made in bioengineering, the future of bacteriophage use in bacterial detection is boundless and limitless.

Table 1: Comparison among Traditional plating techniques, Molecular diagnostic methods, and Bacteriophage-based detectors

	Traditional Plating Technique	**Molecular Diagnostic Methods**	**Bacteriophage-Based Detectors**
Real time diagnosis	No	Yes	Yes
Average diagnostic turn-around time	10-24 hours	More than 24 hours	Less than 24 hours
Labour intensive diagnosis	Yes	Yes	No
Technicality of result interpretation	Yes	Yes	No
Ability to detect viable cells.	No	No	Yes
Production cost	Low	Very high	Low
Cost of reagents	High	Very high	Low
Precision level	Low	High	High
Sensitivity level	Low	High	High
Presence of off-target hits	Yes	Yes	No
Interference by environmental contaminants	Yes	Yes	No

Table 2: Examples of reporter bacteriophages used in bacterial detectors

Engineered reporter bacteriophage	**Reporter gene**	**Target organism**	**Detection method**	**Reference**
A511::*nluc*	Nluc	*Listeria* spp.	Bioluminescence	[27]
A006::*nluc* ΔLCR	Nluc	*Listeria* spp.	Bioluminescence	[27]
A500::*nluc* ΔLCR	Nluc, Rluc, Gluc, LuxAB	*Listeria* spp.	Bioluminescence	[27]
Φ^{2}GFP10 (TM4-derived)	mVenus GFP	*Mycobacterium* spp.	Fluorescence	[41]
phiV10lux	LuxA, LuxB	*E. coli*	Bioluminescence	[42]
HK620	LuxA, LuxB	*E. coli*	Bioluminescence	[43]
Wβ::luxAB-2	LuxA, LuxB, (spc^R)	*Bacillus anthracis*	Bioluminescence	[44]
Y2	LuxAB	*Erwinia amylovora*	Bioluminescence	[45]
Φ^{2}DRMs (TM4 derived)	mVenus GFP, tdTomato RFP	*Mycobacterium* spp.	Fluorescence	[46]
T7$_{LacZ}$	*β*-galactosidase (LacZ operon)	*E. coli*	Electrochemistry	[47]
T7$_{NLC}$	NLuc-CBM2a	*E. coli*	Bioluminescence	[48]
T7$_{LacZ}$	*β*- galactosidase (LacZ operon)	*E. coli*	Colorimetry	[49]
Φ^{2}GFP12 (DS6A-derived)	mVenus GFP	*Mycobacterium tuberculosis*	Fluorescence	[50]
NRGp6 (T7)	NLuc-CBM2a (cellulose binding)	*E. coli*	Bioluminescence	[51]
NRGp7 (T7)	ALP-GBP: gold-binding peptide fusion	*E. coli*	Electrochemistry	[52]

Conclusion

It has become evident that the frontiers of bacterial diagnosis are beyond the scope of the conventional diagnostic methods in use. Biotechnological design of real-time and cost-effective bacterial diagnostic techniques based on the use of fluorescent reporter bacteriophages is not only experimentally conceivable but achievable, even as routine laboratory techniques. If the current trends in biotechnological revolution are anything to go by, the coming years would witness a drastic shift from conventional diagnostic methods, with phage-based detectors most likely at the forefront.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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