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Review

The Application of Bacteriophage Diagnostics for Bacterial Pathogens in the Agricultural Supply Chain: From Farm-to-Fork

Helen J. Jones, BSc (Hons), PhD, Christopher G. Shield, BSc (Hons), MSc, and Benjamin M.C. Swift, BSc (Hons), MRes, PhD

Abstract

Bacteriophages (phages) have great potential not only as therapeutics but as diagnostics. Indeed, they have been developed and used to diagnose and detect bacterial infections, primarily in human clinical settings. The ability to rapidly detect and control bacterial pathogens in agriculture is of primary importance to maintain food security, improve animal health, and prevent the passage of zoonotic pathogens into the human population. Culture-based detection methods are often labor-intensive, and require further confirmatory tests, increasing costs and processing times needed for diagnostics. Molecular detection methods such as polymerase chain reaction are commonly used to determine the safety of food, however, a major drawback is their inability to differentiate between viable and nonviable bacterial pathogens in food. Phage diagnostics have been proven to be rapid, capable of identifying viable pathogens and do not require cultivation to detect bacteria. Phage detection takes advantage of the specificity of interaction between phage and their hosts. Furthermore, phage detection is cost effective, which is vitally important in agricultural supply chains where there is a drive to keep costs down to ensure that the cost of food does not increase. The full potential of phage detection/diagnostics is not wholly realized or commercialized. This review explores the current use and potential future scope of phage diagnostics and their application to various bacterial pathogens across agriculture and food supply chains.

Keywords: bacteriophage, diagnostics, agriculture

Introduction

The increasing scale of production and global distribution of food goods makes quick and accurate microbial detection essential to ensure the circulation of high-quality and safe foods.[1] Reliable, low-cost, and rapid pathogen detection methods are vitally important for the agricultural industry. Although there have been advances within the agricultural sector to develop and use novel tools to detect contaminants, the infrastructure requirements and drive for low costs mean that adopting these new techniques can be difficult. Detection methods within the agricultural supply chain heavily rely upon bacterial culture, molecular tests such as the polymerase chain reaction (PCR), enzyme-linked immunosorbent assays (ELISA), and other immunological response tests. Culture-based detection is considered to be the gold standard for detection and identification of bacterial pathogens within the agricultural supply chain.[2] However, culture methods lack specificity, sensitivity, and accuracy[3]; especially when culturing fastidious organisms, such as mycobacteria, where inhibitors within sample matrices, slow growth rates, and contamination with other organisms can impact the sensitivity of culture.[4] Culture-based work is also time-consuming, labor-intensive, and requires level 2 or level 3 containment laboratories (depending on the organism being grown). Confirmatory tests (PCR and ELISA) are used alongside culture-based testing, again adding to the time, cost, and labor required to positively identify an organism. Slow or incorrect detection results in serious consequences for animal welfare, the agricultural industry, and public health.[5] It is clear that alternative detection methods are needed which are rapid, specific, and can differentiate between active and past infections. Phage-based detection methods have been developed that fulfill these requirements while keeping costs low and hold the potential for automation.

Pathobiology and Population Sciences, Royal Veterinary College, Hatfield, United Kingdom.

Overview of Current Nonculture Detection Methods

Nonculture molecular methods are gaining popularity within agricultural settings to detect bacterial pathogens.[6] The most commonly used molecular method is PCR, however, direct PCR can be expensive, sensitive to inhibition, and cannot discriminate between viable or dead cells.[3,7] This is of particular importance when measuring the efficacy of food processing methods designed to reduce the viable pathogens. Reverse transcription PCR (RT-PCR) has been developed to overcome difficulties of distinguishing between viable and nonviable cells; such tests amplify RNA, which is a product of active metabolic and cellular processes, and therefore, only recently alive organisms can be detected.[7,8] The effectiveness of RT-PCR has been disputed due to increased false-positive results when compared with culture and other methods.[9] Nevertheless, despite technical difficulties, the need to differentiate active from past infection means that these methods have gained substantial commercial interest and development.

Immunological methods such as ELISA's use antibodies to detect proteins or antigens so that pathogens and associated toxins can be identified. However, ELISAs can lack sensitivity and can cross react with similar organisms.[10] Furthermore, both PCR and antibody diagnostic methods require a high level of skill and a laboratory infrastructure.

Whole-genome sequencing (WGS) is an exciting prospect in detection markets. However, the routine use of WGS as a detection tool, especially in agriculture, is prohibitively expensive for food sector industries.

Application of Bacteriophages As Diagnostic Tools

The application of bacteriophages (phage) as diagnostic tools holds promise across numerous sectors, including the medical, food, and agricultural industries.[3] Phages have evolved to infect and propagate within specific bacterial hosts. During infection, phages replicate at a faster rate than bacteria, resulting in a large number of progeny, and in the case of lytic phage, efficient lysis of their host. Therefore, using phage for contaminant detection can lead to increased efficiency, sensitivity, and specificity of testing when compared with PCR and antibody-derived methods within industrial sectors.[11–13] Phage detection platforms have been developed that utilize both lytic and lysogenic life cycles of phage.

The abundance of phages and our flexibility to select for either specific or broad host-range phages have been exploited for several applications, including for therapeutics, bioremediation,[14,15] and in research tools, where phages have been used to identify and differentiate bacterial species since the 1950s.[12] Reasons for the popularity of such phage-based detection methods are that they are low cost, portable, and accessible to a wide range of users, without the need for extensive training or equipment. As such, phage-based methods have the potential to be used throughout the agricultural supply chain—from farm-to-fork—to revolutionize the detection of bacterial pathogens. The progress of commercializing phage technology has been hampered by a lack of coordination and concerted effort between academic, commercial/industry, and regulatory sectors, resulting in the slow adoption of the research and development that has been carried out.

Bacteriophage Detection Methods

Phage detection methods enable the rapid and specific identification of viable bacterial cells, all due to the fact that phages only infect viable hosts. Phages have been used as detection tools in numerous ways to modify, lyse, isolate, and extract their bacterial hosts to enable detection. These methods broadly fall into ''reporter phage'' (Fig. 1a), ''phage amplification'' (Fig. 1b), and ''phage capture'' (Fig. 1c).

Reporter phages are genetically modified to enable the passage of reporter genes into the bacterial host where the gene is expressed, a signal detected, and the pathogen identified. Reporter genes that encode luciferases or fluorescent proteins have been inserted into the phage genome to enable expression of the gene when the phage infects viable hosts[1] (Fig. 1a).

Phage amplification assays use the production of progeny phage or the death of the bacterial host as a detection signal.[1,2] Commonly, the growth of the phage is measured by the formation of plaques on a Petri dish. Plaques are formed when infected hosts lyse releasing progeny phage allowing reinfection of bacteria. Phage amplification requires infection with phage followed by chemical inactivation of extracellular phage using ''virucide,'' and the subsequent detection of plaques using a fast-growing ''reporter'' organism/lawn. Each plaque is considered to be representative of one bacterium originally infected. Plaques can be subsequently extracted and tested for added specificity using PCR[16,17] (Fig. 1b).

Phage capture utilizes attributes of phage, such as endolysins or tail spikes to selectively bind bacteria. Endolysins are enzymes that are produced by phage to break down bacterial cell walls during lysis; they comprise two domains, one catalyzes cell wall breakdown and the other is a cell wall binding domain (CWBD), which specifically recognizes areas of their host cell wall. Phage tail spikes selectively attach and bind to host cells to allow infection. Tail spikes have been utilized to capture specific bacteria from a range of matrices, including food and processing equipment (Fig. 1c).

This review introduces how bacteriophages have been used to detect different pathogens relevant to the agricultural industry from farm-to-fork. As such, we briefly contrast conventional diagnostics with phage-based approaches to detect a range of zoonotic pathogens that cause economic and social hardship.

Detecting Mycobacteria

Mycobacterial species have significantly affected livestock mortality and the economics of production. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and *Mycobacterium bovis* are the two most common mycobacterial pathogens in cattle where they cause Johne's disease and bovine tuberculosis (bTB) and significantly impact the economics and animal welfare worldwide. Annual cost estimates for Johne's disease range from $200 to $250 million across the United States.[18] The estimated annual cost of bTB in the United Kingdom is £120 million due to both governance investment and the direct impact on industry.[19]

The drive for phage-based detection methods is most prominent for mycobacterial infection compared with other infections due to hurdles in the study and understanding of these infections using traditional methods. Phage-detection represents an opportunity to overcome these technical hurdles and provides a platform to apply these techniques to other pathogens.

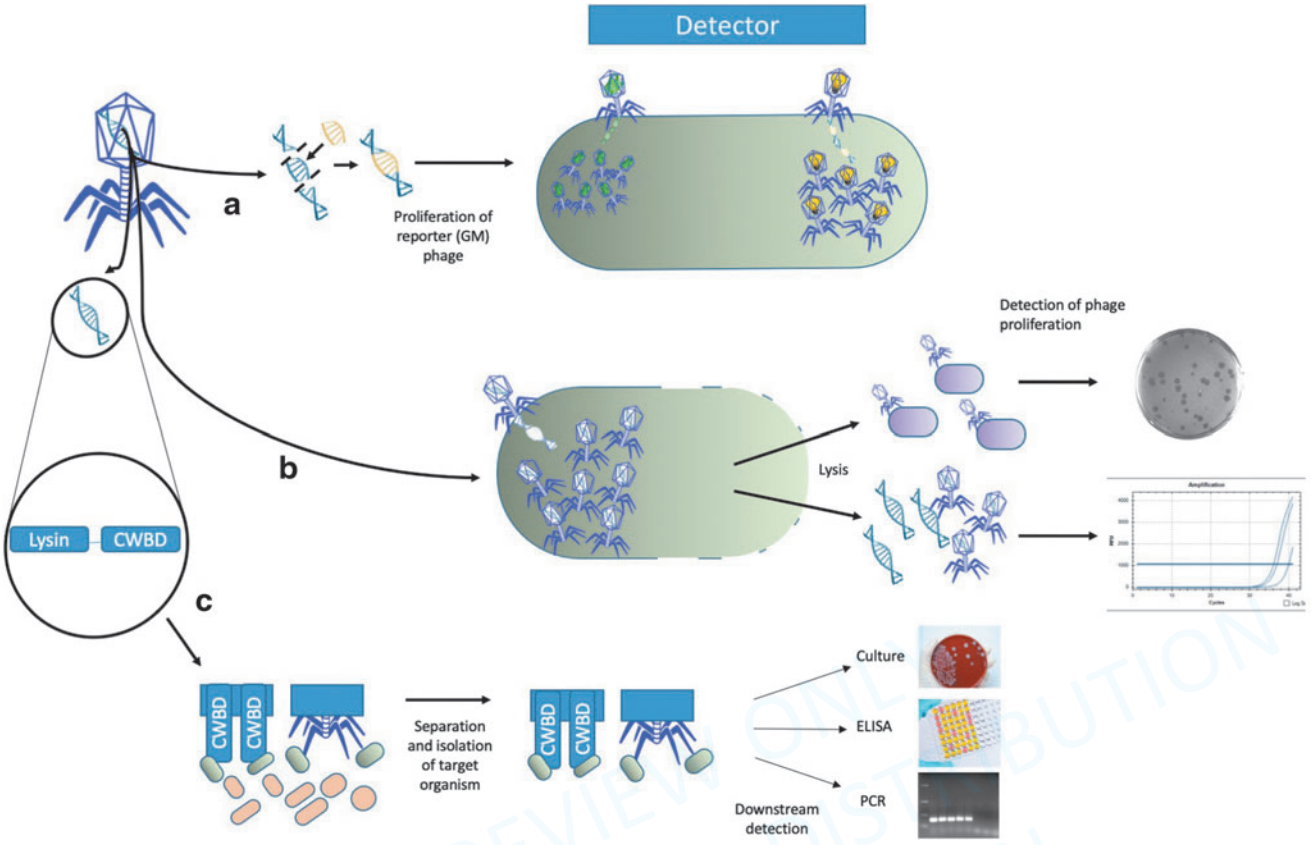

FIG. 1. The application of bacteriophage to diagnostic procedures. **(a)** Reporter phage: the genetic modification of phage with reporter genes to express signals after phage infection and proliferation. Green phage: green fluorescent protein. Yellow bulb: luciferase gene. **(b)** Phage amplification: infection and proliferation of phage followed by downstream detection of progeny phage by plaques on a lawn of host organism, or by qPCR. **(c)** The utilization of cell wall binding domains, or immobilized phage/tail spike to capture and isolate target organisms via downstream separation and detection methods such as culture, ELISA, or qPCR. CWBD, cell wall binding domain; ELISA, enzyme-linked immunosorbent assays; GM, genetically modified; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction.

Johne's disease is a chronic wasting disease affecting cattle and other ruminants; it causes reduced milk yield and weight loss leading to reduced trade value, as well as fertility complications and compromised immunity to other diseases.[20] Exposure to MAP in dairy products has been linked to Crohn's disease in humans, however, the association has not been conclusively determined.[21,22]

bTB is a respiratory infection that can be transmitted to humans through contact with infected animals via respiratory droplets or consumption of unpasteurized dairy products.[23] The human bTB disease burden is likely to be underestimated, especially in low-middle-income countries.[24] Both Johne's and bTB are difficult to detect, due to the subclinical nature of the diseases and the pathogen's ability to evade the host's immune system.

Culture-based approaches are notoriously insensitive and unreliable for diagnosing mycobacterial diseases. Mycobacteria can take several weeks to grow, rendering them susceptible to contamination and making culture impractical as a detection tool to ensure animal health and welfare, and food security.[25]

Immunological-based tools exist for diagnosing both Johne's disease and bTB. Current diagnostic tests for Johne's disease rely on blood or milk ELISAs, which are cheap and can be used in a high-throughput capacity. However, their sensitivity is extremely poor during early stages of infection, allowing the disease to spread among the herd before being diagnosed.[26] The Single Intradermal Comparative Cervical Tuberculin (SICCT) test and the gamma-interferon test are the most widely used tests to diagnose bTB. The SICCT test, although excellent as a screening tool at herd level, is extremely poor at an individual level. A lack of sensitivity means that infections in herds can persist for a long time on farms. Conversely, the gamma-interferon test is sensitive but not specific, resulting in the potential removal of uninfected animals and herd restrictions, which is both economically and emotionally damaging to farmers.[27] Molecular methods exhibit a high degree of variability in sensitivity due to potential inhibitors in the sample matrices and difficulties in lysing mycobacteria, which have a thick waxy cell wall.[20] Inefficient DNA extraction results in insensitive PCR detection of the pathogens. PCR cannot indicate the effectiveness of food processes designed to remove pathogens such as pasteurization as it is insensitive to organism viability.[25,28]

There is a need for diagnostic tests to be cost effective, timely, and to directly detect the intended pathogen rather

than indirectly detect the presence of pathogens based on immunological tests[29]; this has enabled the development, application, and commercialization of phage detection methods.

Mycobacteriophage Amplification

Mycobacteriophage amplification methods (Fig. 1b) have been developed to detect *Mycobacterium tuberculosis* (causative pathogen of human tuberculosis [TB]) in human sputum samples.[30] This technology was commercialized as the FASTPlaqueTB assay (Biotec Laboratories) and now available as the rebranded Actiphage® Core assay (PBD Biotech, UK). These assays used mycobacteriophage D29, a broad-spectrum phage capable of infecting a range of mycobacteria. A PCR step was developed using DNA from plaques formed at the end of the assay to improve the specificity of the assay in agricultural settings.[31] The assay reporting time was 24–48 h with a limit of detection of 1–10 plaque forming units per mL. This assay has been deployed in a variety of matrices targeting both MAP and *M. bovis* from farm-to-fork settings; the following describes how and where.

In farm settings, the phage amplification assay has been used on both milk and blood samples. Testing was carried out on bulk milk tank (BMT) samples to establish the effectiveness of phage diagnostics and prevalence of MAP in herds.[31] The phage amplification assay was used on BMT samples from a Johne's infected herd. Botsaris et al.[22] detected viable MAP in 22% of BMT samples compared with just 0.9% by culture during research and development investigations ($n=225$; Table 1). In later work, a positive correlation was established between plaque number and the likelihood of a milk sample being positive for MAP without the need for the plaque-PCR, demonstrating high sensitivity (90%) and specificity (99%) in individual cattle[32] (Table 1). Peptide-mediated magnetic separation (PMS) before phage amplification and detection of plaques demonstrated good detection of MAP in 26% of BMT samples compared with direct fecal quantitative polymerase chain reaction (qPCR) and culture.[33] This method was applied to milk from Johne's infected farms and proved to be more sensitive compared with PMS combined with direct-qPCR and PMS combined with culture[34] (Table 1). PMS plus phage amplification was found to have a sensitivity of 32% and 100% specificity, an improvement on culture (Table 1), but not sensitive enough for practical use.[35] Phage capture has been used to detect MAP in BMT by fusing D29 phage to tosylactivated paramagnetic beads combined with qPCR testing; the limit of detection is reported as 10 MAP cells/50 mL of milk.[36]

Magnetic separation was also incorporated into the phage amplification assay to isolate MAP cells from peripheral blood mononuclear cells[25,37] (Table 1). Technology transfer and optimization of the protocol caused variability in testing procedures; the magnetic separation step was thought to limit the sensitivity of the assay in blood samples.[37]

The phage amplification method has also been used to detect *M. bovis* in blood samples. Instead of using PCR, recombinase polymerase amplification was combined with the phage amplification assay to test for *M. bovis* in the blood of SICCT-positive and SICCT-negative cattle. It was shown that 66% ($n=41$) of TB-infected cattle (based on positive SICCT result) tested positive for viable *M. bovis* in blood. The negative control herds all tested negative by the phage amplification assay[38] ($n=45$).

Recently, the phage amplification assay has been further developed removing the need for Petri dishes and overnight incubation for use as a novel platform technology called Actiphage Rapid[39] (PBD Biotech). Actiphage Rapid reduces the reporting time from 24–48 to 6 h. This format of the assay relies on the bacteriophage to act as a DNA lysis reagent, efficiently releasing mycobacterial DNA from viable cells, allowing detection using PCR. The sensitivity of phage diagnostics for mycobacteria using Actiphage Rapid has been dramatically improved whilst not compromising specificity (Table 1). The Actiphage assay has been used to detect both *M. bovis* and MAP in blood samples.[39] The reduction in time and increase in sensitivity hold promise for the future of farm testing of mycobacterial pathogens using phage diagnostics.

In food samples, the effectiveness of pasteurization in eliminating MAP from retailed milk products has been questioned.[28,40,41] The phage amplification assay has been used to detect viable MAP in food products, such as milk and cheese.[21,22,42,43] Viable MAP was detected in 10% ($n=368$) of milk products from supermarkets (Table 1). Evidence of viable MAP has also been found in desiccated milk products such as calf milk replacer[43] and powdered infant formula (Table 1).[21] MAP was detected in cheese (Table 1), indicating the ability of this pathogen to survive for a long time at low pH.[22] These studies demonstrate that MAP may survive through food processes such as pasteurization and desiccation, resulting in contaminated food products on the shelf.[28]

This existing technology can be implemented within farm, dairy processing, and product testing settings, where culture is unsuitable or not sensitive enough, thereby improving disease control and surveillance within farm settings, providing greater information about the effectivity of pasteurization processes, and the extent of the exposure of the public to mycobacterial pathogens.[22,27,28] The high costs associated with the burden and elimination of mycobacterial pathogens in agricultural settings and the acknowledgment that detection methods require improvement have enabled the commercialization and application of phage detection. These attributes facilitated greater coordination between academic, industrial, clinical, and regulatory sectors. This will hopefully enable future development and application of phage detection methods to other pathogens to progress more rapidly and effectively.

Listeria monocytogenes

Listeriosis affects between 0.1 and 10 people per million globally; 90% of cases result in patients being hospitalized and a mortality rate of 20–30%.[44,45] It is a foodborne disease associated with preprepared ready-to-eat products such as meat, fish, and diary. Risk factors include immunocompromised individuals, pregnancy, old age, and diseases such as HIV/AIDS and cancer.[46] Mild symptoms include gastroenteritis, fever, diarrhea, nausea, vomiting, and abdominal pain. Severe complications can lead to meningitis or sepsis.[47] Listeriosis is a significant public health concern, but if diagnosed early can be treated using antibiotics.[46]

L. monocytogenes is ubiquitously found in natural environments and animal hosts,[47] and can affect livestock and wildlife populations.[48] The pathogen can impact various points throughout the agricultural supply chain by virtue of being able to survive and propagate in conditions used to

Table 1. Mycobacteriophage Detection/Diagnostic Studies for *Mycobacterium avium* subsp. *paratuberculosis* and Bovine Tuberculosis, from Farm-to-Fork

Reference	*Sample matrix*	*Organism detected*	*Results*	*Diagnostic method*
Stanley et al.[31]	Bulk tank milk	MAP	Phage PCR, 67%, $n=15$	Phage detection-plaque PCR
Botsaris el al.[22]	Bulk tank milk	MAP	Phage-PCR, 22.2%, $n=225$; qPCR (IS900), 28.6%, $n=220$; culture, 0.9%, $n=225$	Phage detection-plaque PCR
	Cheese	MAP	Cheese: qPCR, 25%, $n=28$; phage-PCR, 0%, $n=14$	
Foddai et al.[33]	Bulk tank milk	MAP	PMS-phage, 40%; culture, 12%; $n=25$ (Irish cattle); PMS-phage, 26%; culture, 11%; $n=19$ (American cattle)	PMS-phage
Botsaris et al.[32]	Bulk tank milk	MAP	Phage-PCR, 22% $n=225$; culture, 0.9%, $n=225$. 90% sensitivity and 99% specificity	Phage detection-plaque PCR
Swift et al.[25]	Blood	MAP	Sensitivity, 100%, $n=9$ (measured against blood and milk ELISA); specificity, 100%, $n=5$. Mixed status herd: phage-PCR, 80%; milk ELISA, 60%; blood ELISA, 40%; culture, 0%; $n=10$	PMMS-phage, phage PCR
Botsaris et al.[21]	Powder infant formula	MAP	Powder infant formula: phage-PCR, 13%; culture, 9%; qPCR, 22%; $n=32$	Phage detection-plaque PCR
Swift et al.[38]	Blood	*Mycobacterium bovis*	66% sensitivity (SICCT-positive animals), $n=41$; 100% specificity (SICCT-negative animals), $n=45$	Phage-RPA
Swift et al.[37]	Blood	MAP	PMMS-phage assay (blood), 37%; culture (fecal), 11%; PCR (fecal), 11%, serum AB ELISA (positive or suspected), 11%; $n=19$	PMMS-phage
Foddai and Grant[34]	Individual milk	MAP	PMS-phage, 21.2%, $n=146$; PMS-qPCR (IS900), 9.1%, $n=77$; PMS-qPCR (F57), 2.6%, $n=77$; PMS-MGIT culture, 11.6%, $n=146$	PMS-phage
	Bulk milk tank	MAP	PMS-phage, 59.1%; PMS-qPCR (IS900), 45.5%; PMS-qPCR (F57), 9.1%; PMS-MGIT culture, 50%; $n=22$	
Grant et al.[43]	Calf milk replacer	MAP	PMS-phage assay, 20.5%; PMS culturing, 14.5%; IS900 PCR, 8.4%; $n=83$	PMS-phage
O'Brien et al.[35]	Individual milk	MAP	PMS-phage, sensitivity 32.5%, $n=40$; specificity 100%, $n=105$. PMS-culture, 25% sensitivity, $n=40$; specificity 96.2%, $n=105$	PMS-phage
Gerrard et al.[42]	Supermarket milk	MAP	Phage-PCR, 10.3%; PCR, 3.5%; $n=368$	Phage-PCR
Swift et al.[39]	Blood	*M. Bovis*	Sensitivity 95%, $n=41$ SICCT-positive animals; specificity 100%, $n=45$ SICCT-negative animals	Actiphage® Rapid
Foddai and Grant[36]	Bulk tank milk	MAP	PhMS-qPCR, 49%, $n=100$	Tosylactivated paramagnetic beads coated in phage (PhMS), qPCR

ELISA, enzyme-linked immunosorbent assays; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; MGIT, mycobacteria growth indicator tube; n, number of samples; PCR, polymerase chain reaction; PhMS, phagomagnetic separation; PMS/PMMS, peptide-mediated magnetic separation; qPCR, quantitative polymerase chain reaction; RPA, recombinase polymerase amplification; SICCT, single intradermal comparative cervical tuberculin.

preserve food such as low pH, low temperature, and high salt. The products most prone to *L. monocytogenes* contamination are those associated with minimal processing procedures. *L. monocytogenes* exhibits biofilm formation, which further enables persistence within factory settings.[47]

L. monocytogenes detection methods have been implemented both within farm and factory settings. Culture of the organism is considered to be the gold standard for detection[45] and is sometimes combined with PCR or immunoassays to increase specificity.[47] *L. monocytogenes* phage-based detection can confer an increase in speed compared with culture (up to 72-h reduction in processing time).[49] A further advantage of phage methods is that they specifically detect viable organisms unlike molecular techniques that might identify heat-inactivated/pasteurized microorganisms as potential pathogen contamination, resulting in reporting false contamination.

Phage amplification assays (Fig. 1c) have been developed for *Listeria* using the broad host-range listeriophage, A511. A plaque detection model was developed and tested using inoculated milk; the limit of detection was 13 colony forming units (cfu)/mL with a reporting time of 10 h.[50] Phage detection has also been coupled to surface-enhanced Raman spectroscopy and lateral flow immunochromatography to develop a quantifiable reporter system; the limit of detection was between 5×10^4 and 6×10^6 cfu/mL.[51]

Reporter phage diagnostics (Fig. 1a) have also been developed for *Listeria*. The luciferase gene has been incorporated into the Listeria-specific A511 phage, resulting in a bioluminescent phenotype after viable host infection.[52] Detection of 10^2–10^3 cells was achievable after a 2-h incubation period; sensitivity could be increased to 1 cell per gram of artificially inoculated food produce when a pre-enrichment step was applied. This method reduces reporting time, although limited by a lack of sensitivity (without the need of enrichment) and an inability to differentiate within the *Listeria* genus, although the generation of phage mutants for specific hosts is proposed to overcome this problem.[52] The A511::*luxAB* phages were tested on naturally contaminated food and environmental samples using a 20 h pre-enrichment step. The reporter phage method reported that 15.8% of all samples exhibited *L. monocytogenes* contamination compared with 16.4% using standard plating methods across raw meat, poultry, dairy products,and plant samples (Table 2).[49] Numerous luciferase reporter phages have been tested using *de novo* genome assembly/activation and CRISPR-Cas phage engineering.[45] Luciferase phage reporter systems demonstrated similar sensitivity to standard culture; however, the phage assay detection time was 24 h in contrast to 96 h by culture.[49]

The luciferase recombinant reporter phage has been commercially adapted as Sample 6 Detect HT/L and was certified by the Association of Official Agricultural Chemists for the detection of *Listeria.*[11] Originally developed to test environmental samples, Sample 6 Detect HT/L has been optimized to identify 50 Listeria isolates, and to differentiate from 30 non-Listeria strains that do not cause contamination in industrial food processes.[11,53] The kit was marketed at $16 for a 10 sample kit in 2016[11]; the assay's reporting time is 6 h and therefore holds potential for production line microbial detection. The Sample 6 Detect HT/L has been recently verified for testing produce such as ice cream,[54] leafy greens,[55] and environmental stainless-steel surfaces,[56] with results comparable with culture methods commercially used and a reduced reporting time. *Listeria* phage detection holds the potential to improve on gold standard methods in both farm and food processing infrastructures in a variety of matrices. The Sample 6 Detect HT/L is currently commercially available.

The phage capture method (Fig. 1c) has also been applied to phage diagnostics for *L. monocytogenes*. The phage-derived CWBD protein has been successfully fused to paramagnetic beads (cell wall binding domain proteins and magnetic separation [CBD-MS]) followed by either culture or PCR. CBD-MS was challenged with the contaminated substance and successfully extracted *Listeria* (Table 2); it increased sensitivity and relative performance compared with standard plating techniques both in artificially and naturally contaminated foods.[57] The phage capture method using CWBD has been combined with plate count and qPCR.[58] The reporting time is improved compared with standard culture procedures, although there was a poor limit of detection (Table 2).[58] Phage capture using CBD-MS has been combined with luciferase reporter phages so that capture and detection can be efficiently combined to be rapid and specific. The method provided high sensitivity and a reduced reporting time compared with culture (Table 2).[59] In addition, fluorescent marker proteins have been fused to the CWBD to extract and identify specific Listeria serotypes using magnetic separation. Although the reporting time is reduced, the sensitivity was not tested or reported.[60] Phage CWBD has also been immobilized onto printed electrodes and used as an electrochemical impedance biosensor, which can be integrated into an automated platform, however, the limit of detection is currently too high for practical application (Table 2).[61]

Salmonella spp.

Salmonellosis is caused by ingestion of *Salmonella* on contaminated food or products. Symptoms include gastroenteritis, fever, abdominal pain, diarrhea, and vomiting. Surveillance of agricultural supply chains suggests that *Salmonella* contamination occurs at low levels, thus implying that low exposure can cause the disease; fewer than 10 *Salmonella* cells can cause salmonellosis.[2] *Salmonella* can be contracted from both crops and animal produce.[62] The economic and social cost of *Salmonella* food poisoning is thought to be £210 million in the United Kingdom.[63] Outbreaks are normally detected in point-of-care health settings, although laboratory testing is conducted throughout the agricultural food chain.[64] Again, *Salmonella* culture is considered the gold standard,[2] however, long culture times clearly reduce the impact of testing in food outbreak scenarios that require rapid intervention to prevent further outbreaks. PCR has been developed to detect *Salmonella* in contaminated foods, but is insensitive and requires pre-enrichment steps, thus further increasing reporting time.[2] Mass spectrometry and next-generation sequencing-based methods have been considered for commercial salmonella diagnostics,[2] however, there are technical limitations associated with both approaches and start-up costs may be too high for industrial application.

Salmonella phage-based methods have been demonstrated in crops and livestock. Phage amplification,[65,66] reporter

Table 2. Summary of Phage Detection for *Listeria* Pathogens

Reference	*Method of detection*	*Time to reporting*	*LOD*	*Sample type*	*Sample size*
Loessner et al.[52]	Luciferase bioluminescence; A511:luxAB	<3 h	5×10^2–10^3 cells/mL. Pre-enrichment: 1 cell/g of sample	Artificially contaminated salad	20
Loessner et al.[49]	Luciferase bioluminescence; A511:luxAB	24 h	<1 cell/g of food	Ricotta cheese, chocolate pudding, cabbage	348
Kretzer et al.[57]	Paramagnetic beads coated with *Listeria* phage endolysin-derived CBD-MS. Endpoint: viable cell count, PCR	48 h	0.1 cfu/g	Meat, poultry, fish, dairy, and deli items	275
Schmelcher et al.[60]	CWBDs fused to fluorescent marker proteins	15 min	N/D	Artificially contaminated milk or cheese	N/D
Walcher et al.[58]	Paramagnetic beads with phage CWBDs followed by culture or RT-PCR	48 h	10^2–10^3 cfu/mL	Artificially contaminated milk	N/D
Hagens et al.[3]	ß-glycosidase; A511::*celB*	6 h	<10 cfu/g	Artificially contaminated chocolate milk and salmon	N/D
Tolba et al.[61]	CWBD fused to gold screen printed electrode and detected by electrochemical impedance spectroscopy	<6 h	1.1×10^5 cfu/mL	Artificially contaminated milk	N/D
Kretzer et al.[59]	CBD-MS, A511::*luxAB*	<24 h	0.1–1.0 cfu/g	Iceberg lettuce, chocolate milk, mozzarella cheese, Swiss red smear soft cheese, Swiss white mold cheese, preprepared shrimp, minced meat, smoked salmon, smoked turkey breast	9
Banerjee et al.[56]	Sample 6 Detect HT/L	6 h	N/D	Stainless steel environmental samples	5
Arias-Rios et al.[54]	Sample 6 Detect HT/L	15 h	0.044–0.48 cfu/g	Ice cream	30

CBD-MS, cell wall binding domain proteins and magnetic separation; cfu, colony forming units; CWBD, cell wall binding domain; LOD, limit of detection; N/D, not declared; RT-PCR, reverse transcription PCR.

phage,[67] and phage capture methods[68,69] have been used to detect *Salmonella* (Table 3). However, these studies are research and development focused and not currently available for commercial distribution.

The phage amplification method reduced reporting time from 4 days (traditional culture) to 18 h using the formation of plaques to indicate pathogen presence[65]; the limit of detection (40 cells/mL) was not sensitive enough to detect disease causing contamination[2] (Table 3).

The reporter phage method using luciferase could detect contamination at 20 cfu/mL within 2 h (Table 3).[67] The reporting time is an advantage over gold standard methods, however, the limit of detection requires improvement.[2]

Immobilization of phage to enable extraction and detection has also been developed using whole phage. Magnetic separation has been used by binding and immobilizing wild-type phage on magnetic particles to enable better specificity in subsequent antibody immunoassays.[69] Whole phages have also been immobilized on paper via piezoelectric inkjet printing by mixing phage within inks. Plaque assays or qPCR of phage DNA was used to test pathogen capture abilities of paper-bound phage and consequently used in detection of proof-of-concept tests. The reporting time is between 8 and 26 h, dependent upon the endpoint test[66] (Table 3).

Existing data have focused on testing after food processing, shelf products. Extending testing of *Salmonella* phage diagnostics throughout the manufacturing process and within farm environments would provide valuable data and move the technology toward farm site use. This would enable key insights about where *Salmonella* contaminates and propagates effectively within the agricultural supply chain, enabling control measures to be implemented before food products are created.

Escherichia coli

Shiga toxin-producing *E. coli* are an important group of foodborne pathogens causing an estimated 265,000 illnesses each year in the United States alone.[71] Symptoms include stomach cramps, diarrhea, and vomiting. The infection is usually self-limiting but can be life-threatening. Cattle are considered a major reservoir; contaminated beef, dairy, and salad products frequently cause outbreaks[72] and necessitate a

Table 3. Summary of *Salmonella* Phage Detection Methods

Reference	*Method of detection*	*Time to reporting*	*LOD*	*Sample type*	*Sample size*
Stewart et al.[65]	Phage amplification assay	4 h	6×10^2 cfu/mL	Culture, proof of concept	N/A
Muldoon et al.[70]	Immunochromatographic strip-based detection of *Salmonella* after phage suppression of cross-reactive species	N/D	N/A	Artificially contaminated ground pork, naturally contaminated beef samples	115
Morton et al.[68]	*Salmonella*-specific phage proteins bound to paramagnetic beads for magnetic separation followed by Greenlight detection (testing for metabolic activity)	12–24 h	3×10^1 cfu/mL	Culture, proof of concept	N/A
Kim et al.[67]	Bioluminescent reporter phage, SPC32H-CDABE	2 h	2×10^1–7×10^2 cfu/mL	Artificially contaminated lettuce, sliced pork, and milk	N/D
Annay et al.[66]	Piezoelectric inkjet print binding phage (phage-based bioactive paper strip). Endpoint: RT-PCR of phage	8 h	1–5×10^1 cfu/mL	Spinach, ground beef, and chicken	N/D

N/A, not applicable.

recall of produce. Rapid, sensitive, and affordable *E. coli* detection in the agricultural supply chain is vital for the prevention of foodborne illnesses.

Again, the current gold standard of detection is culture- and PCR-based, but this can take 1 week for results—too slow in an outbreak situation. The current detection method used in agricultural water (Environmental Protection Agency [EPA] 1603)[73] requires highly trained personnel and verification of multiple colonies and takes ~24 h to complete. The minimum infectious dose of *E. coli* is very low, and thus, new detection methods need to be highly sensitive, ideally 1–10 cfu/100 g of food.[74,75] The EPA states that no level of *E. coli* contamination in public water supply is acceptable,[76] methods capable of detecting one cell in a 100 mL sample are needed.

Phage-based detection has several advantages over conventional methods. The remarkable host specificity of phage can be exploited to create highly specific detection methods. However, in many studies, the assay specificity was assumed—not demonstrated.[66,77–81] For full confidence in results, assay specificity should be demonstrated experimentally. Another advantage of phage comes from the rapid amplification inside the host. Evident as all methods presented here were faster than the EPA 1603 method (requiring 24 h) and the gold standard (requiring 1 week). In the fast-paced environment of contaminant detection and outbreak prevention, this advantage cannot be overemphasized.

Numerous approaches have used phage engineered to induce overexpression of luciferase.[80,83,84,86] These were some of the longest to perform assays,[80,83] however, the sensitivity was among the best reviewed here.[80,84,86] The assay developed by Zhang et al.[84] had sensitivity (12.5 cfu/100 mL) acceptably close to the ideal (1–10 cfu/100 g), good specificity, and at $0.20 per assay, this detection platform offers fast and sensitive detection at a price amenable to high throughput (Table 4). Another success came when luciferase/alkaline phosphatase-engineered reporter phage, in conjunction with a cellulose filter, was used to detect 1 cfu/100 mL.[86] Demonstrated to be equally sensitive as the EPA 1603 method (1 cfu/100 mL), but in half the time; however, this assay was not tested in complex matrices and specificity not evaluated.

A number of these studies utilize the narrow host range of phage as an approximation for specificity and neglect to demonstrate the specificity of the assay in practice. Few studies here report an approximate cost—an important metric when determining an assay's suitability. Lastly, many methods utilize expensive equipment that requires specialist personnel, limiting the methods use outside reference laboratories and likely excluding high-throughput use. Despite these limitations, the advantages gained in reporting time of phage over the gold standard and EPA 1603 methods are evident; with continued development and improvements in throughput, usability, and cost, phage-based *E. coli* detection methods will meet the needs of commercial application for the agricultural supply chain.

Crop Disease Diagnostics

A limited number of research and development studies have applied phage-based diagnostics to crop pathogens associated with blight and wilt; these methods are not currently available for commercial distribution.

Existing phage-based diagnostics have been developed for *Pseudomonas*, *Erwinia*, and *Ralstonia*.[87] *Pseudomonas cannabina* pv. *Alisalensis* is responsible for bacterial blight in cruciferous vegetables, *Brassicaceae*, which is nutritionally important and responsible for oil seed and energy crops.[88,89] *Brassicaceae* crops have an estimated value of $1.3 billion in the U.S. trade markets; blight damages these crops resulting in unmarketable products and therefore economic loss. *Erwinia amylovora* causes fire blight in *Rosaceae* plants, including global fruit produce such as apples and pears. The economic costs associated with *Erwinia* blight arise from control measures, compensation costs, and the destruction of infected plants[90]; losses amount to as much as

TABLE 4. SUMMARY OF BACTERIOPHAGE-BASED *ESCHERICHIA COLI* DETECTION METHODS

Reference	*Method description*	*Phage*	*LOD*	*Assay time (hours)*	*Tested matrices*	*Cost per assay (USD)*
Goodridge et al.[82]	Flow cytometric detection of cell fluorescence following immunomagnetic separation	Fluorescently labeled LG1	2.2 cfu/g 101–102 cfu/mL	7 12	Ground beef Raw milk	Not reported
Ripp et al.[83]	Bioluminescent detection of autoinduced signaling molecule	λ_{luxI} (GM)	130 cfu/mL	22.4	Lettuce washings	Not reported
Zhang et al.[84]	Bioluminescent detection of overexpressed luciferase following substrate addition	$\Phi V10_{nlc}$ (GM)	5–6 cfu/40 mL	9	Ground beef	0.20
Anany et al.[66]	Phage dipstick and RT-PCR	rV5	10–50 cfu/mL	8	Spinach, ground beef, chicken	Not reported
Chen et al.[81]	Visual detection of a overexpressed catalytic enzyme product, following colormetric substrate addition	$T7_{lacZ}$ (GM)	100 cfu/mL	7	Drinking water, skimmed milk, orange juice	Not reported
Wang et al.[85]	Electrochemical detection of an overexpressed catalytic enzyme product, following substrate addition	$T7_{lacZ}$ (GM)	100 cfu/mL	7	Drinking water, skimmed milk, apple juice	<10.00
Hoang et al.[77]	Colorimetric detection following *E. coli* selection using antibiotics (using spectrophotometry)	$PP01_{ccp}$ (GM)	2 cfu/g	16.5	Vegetable matrices	Not reported
Hinkley et al.[86]	Colorimetric and bioluminescent detection of phage-induced luciferase and alkaline phosphatase following filtration	$T7_{nlc}$/T7-ALP (GM)	1 cfu/100 mL	10	Drinking water	Not reported
Yang et al.[78]	Fluorescent image analysis of phage lysed *E. coli* (using cell morphology)	T7	10 cfu/mL	8	Fresh produce washings, coconut water, spinach washings	5.50
Kozak and Alcaine[80]	Visual detection of bioluminescence caused by overexpression of a luminescent molecule	$T7_{nlc}$ (GM)	2.37 cfu/well 5.48 cfu/well	>24 >22	Incorporated within cheese Topically over cheese	Not reported
Wisuthiphaet et al.[79]	Detection of reporter enzyme using an additional substrate followed by fluorescent imaging	T7-ALP (GM)	100 cfu/mL	6	Coconut water, apple juice	Not reported

All tested matrices were artificially contaminated.
cfu, colony forming unit; GM, genetically modified; $phage_{nlc}$, NanoLuc luciferase; $PP01_{ccp}$, cytochrome c peroxidase; T7-ALP, alkaline phosphatase; $T7_{lacZ}$, β-galactosidase; λluxI, luciferase.

Table 5. Summary of Crop Pathogen Phage Diagnostic Testing

Reference	*Target organism*	*Method of detection*	*Time to reporting*	*LOD*	*Sample type*	*Sample size*
Schofield et al.[88,89]	*Pseudomonas cannabina* pv. *alisalensis*	Bioluminescent phage, PBSPCA1	2 h	1.3×10^3 cfu/mL	Artificially infected plant leaves	N/D
Born et al.[90]	*Erwinia amylovora*	Bioluminescent phage, Y2::*dpoL1-C*, Y2::*luxAB*	<1 h	3.8×10^3 cfu/mL	Field isolates displaying symptoms	24
Kutin et al.[92]	*Ralstonia solanacearum*	Phage amplification and RT-PCR	1 h	10^2 cfu/g	Leaf tissue and soil	N/D

4 million EUR per annum (EU).[91] *Ralstonia solanacearum* is the causative agent for bacterial wilt in over 50 species of tropical agricultural crops and adapted to potatoes and tomatoes in temperate climates.[92] *Ralstonia* wilt costs the potato industry up to $1 billion per annum and can cause up to 90% loss of yield in tomato crops.[93] Current diagnostics are confined to culture methods, visual inspection of specimens, immunological testing, and PCR of crops within farm settings[90]; these methods might lead to a lack of sensitivity and underreporting due to other causes of blight/wilt symptoms such as fungal infections of crops. Phage diagnostics may enable a cheaper alternative that delivers a more rapid result thus enabling sensitive and specific identification of disease, supporting trade and reducing quarantine times.

Phage isolated and extracted using *Pseudomonas cannabina* pv. *Alisalensis* has been used to species type and identify *Psuedomonas*.[94] Reporter phage diagnostics for *Pseudomonas cannabina* pv. *Alisalensis* using a luciferase recombinant phage PBSPCA1 have since been developed and tested (Table 5).[88,89] The reporter phage can further differentiate between *Pseudomonas cannabina* pv. *Alisalensis* and *Pseudomonas syringae* pv. *Maculicola*, a different plant pathogen.[88,89] Phage diagnostics for *E. amylovora* also use luciferase within a reporter phage context, engineering the Y2 phage (Table 5); sensitivity and specificity are improved in naturally infected environmental samples compared with ELISA-based methods.[90] Phage diagnostics for *R. solanacearum* use a phage amplification detection method combined with real-time PCR of phage DNA (Table 5).[92] The method proves to be more sensitive and less destructive compared with existing methods. This area of research is ripe for continued investigation to reduce diagnostic costs and reporting times within the agricultural industry.

Future Perspective and Conclusions

Phage detection/diagnostic methods, when applied to the agricultural supply chain, have consistently been demonstrated to show significant potential to reduce reporting times compared with the existing gold standard methods. Their utilization could produce substantial economic gains by preventing and eliminating the spread of disease and aid in the early identification of contamination, thus avoiding recalls of produce across farm, manufacturing, and shelf testing contexts. Sensitivity and specificity have been shown to be comparable with gold standard techniques, if not better.[25,32–34,49] Phage technology, using phage propagation and reporter phages, has already been commercialized and is currently being distributed by PBD Biotech and Sample 6 Detect for *Mycobacterium* and *Listeria* detection, respectively. The take-up of these technologies is currently in the early stages and is not routinely established. Work is required for validation of these phage technologies and a wider uptake, but a lot of effort has been put into demystifying phages and exploring their role as detection agents.

Currently, there is a drive to create greater control of antibiotic usage within agricultural systems to reduce the selection for antimicrobial-resistant pathogens. Phages are being proposed as a replacement for antibiotics,[14,15] a phage-based detection system may complement this technology, however, there needs to be careful consideration in designing endpoint signals (treatment versus detection). This review has examined a selection of current evidence and development of phage-based diagnostic methods used in the agricultural supply chain. Synthetic biology and phage genome engineering hold exciting prospects for the reprogramming of phage host ranges, function, and isolating functional proteins from phage; these advances will enable synthetic manufacture and specific control of phage development for detection/diagnostic application. Phage-based methods could benefit from automation to reduce human sample handling error and enable point-of-use application. The combination of synthetic biology and automation of sample processing, detection, and reporting is a major step toward biosensor development.[95–97] A biosensor with integrated phage detection enables the advantages of phage-based strategies to be applied and performed away from laboratory environments and further reduce reporting times while tracking pathogen transmission in real time. Real-time identification of spread of disease and supply chain contamination through the application of phage detection/diagnostics would prevent expensive procedures such as product recall and extensive contamination investigations, and potentially save lives.

Authors' Contributions

All authors contributed to the research and writing of the article; all authors read and approved the final article.

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Author Disclosure Statement

The authors declare that the Actiphage technology described in this review has been patented (Rees and Swift; PCT/GB2014/052970; European patent No. EP 3052650; U.S. Patent 10,344,339) and BMCS is a Director at PBD Biotech Ltd., the company commercializing the Actiphage technology.

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Address correspondence to:
Helen J. Jones, BSc, PhD
Pathobiology and Population Sciences
Royal Veterinary College
Hawkshead Ln
Hatfield AL9 7TA
United Kingdom

Email: heljones@rvc.ac.uk