Bacteriophage based diagnostics for on-farm bovine mastitis testing

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Abstract

Bovine mastitis remains one of the most costly and persistent diseases in the global dairy industry, causing annual losses of up to 32 billion USD through reduced milk yield, treatment costs, and premature culling. Current diagnostic tools – such as somatic cell counts, microbial culture, and PCR – are hindered by slow turnaround times, high costs, and technical demands, leading many producers to default to broad antibiotic use. This practice fuels antimicrobial resistance and limits targeted disease control.

Bacteriophage-based diagnostics offer a promising alternative. Phages, viruses that specifically infect bacteria, provide highly selective detection of mastitis-causing pathogens such as *Staphylococcus aureus*, *Streptococcus uberis*, and *Escherichia coli*. Reporter phages genetically engineered to express measurable signals upon infection can deliver rapid, pathogen-specific results within minutes. Lyophilization enhances reagent stability, while colorimetric reporter systems enable simple, visual interpretation suitable for on-farm use.

Beyond mastitis, phage technologies are already established and applied in food processing as antimicrobial washes to reduce *Listeria monocytogenes*, *Salmonella spp.*, and *E. coli* O157:H7 contamination. Extending this proven safety record to diagnostic applications could improve surveillance throughout the dairy supply chain.

By combining precision, speed, and field applicability, bacteriophage diagnostics represent a practical and sustainable advancement in veterinary and food safety microbiology. Continued development of multiplex assays, field validation, and regulatory frameworks will be critical for translating this technology into routine farm and processing practices.

Table of Contents

List of Tables

List of Abbreviations

USD: United States Dollar

IMI: Intramammary infection

PCR: Polymerase chain reaction

SCC: Somatic cell count

CMT: California mastitis test

SFMT: Surf field mastitis test

SPC: Standard plate count

CFU/mL: Colony forming units per milliliter

Ct: Cycle threshold

AMR: Antimicrobial resistance

LAMP: Loop-Mediated Isothermal Amplification

MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization-Time of Flight

CPRG: chlorophenol red-β-D-galactopyranoside

GRAS: Generally Recognized As Safe

USDA: United States Department of Agriculture

CVB: United States Department of Agriculture Center for Veterinary Biologics

LOD: Limit of detection

RBP: Receptor binding protein

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

QC: Quality control

MOI: Multiplicity of infections

GMP: Good Manufacturing Practice

FDA: Food and Drug Administration

Introduction

Bovine mastitis is one of the most prevalent and economically devastating diseases in the global dairy industry, responsible for substantial production losses, increased veterinary costs, and premature culling of affected animals (1–3). The disease, characterized by inflammation of the mammary gland, can result from a variety of factors, but intramammary infections (IMIs) caused by bacterial pathogens such as *Staphylococcus aureus*, *Streptococcus uberis*, and *Escherichia coli* represent the most common and clinically significant cause (1, 4). Despite advances in understanding mastitis pathophysiology, current diagnostic and management practices remain inadequate to prevent widespread and recurrent infection (2, 5, 6). The diagnostic process often relies on indirect indicators of inflammation, such as somatic cell counts or cow-side tests, with confirmatory identification of the causative organism rarely performed in the field (2, 4, 5, 7). This gap between detection and pathogen identification perpetuates the use of broad-spectrum antibiotics as a default treatment, contributing to antimicrobial resistance and posing growing challenges to both animal and public health (2, 6).

The continued overreliance on empirical antibiotic therapy emphasizes the need for rapid, accurate, and accessible diagnostic systems that can distinguish between mastitis-causing pathogens directly on the farm. Conventional methods, such as microbial culture and polymerase chain reaction (PCR), provide reliable results but are constrained by long turnaround times, high costs, and the need for specialized laboratory infrastructure (2, 4, 7). The limitations create a diagnostic bottleneck that hinders effective disease management and undermines efforts to promote improved antimicrobial oversight within the dairy sector.

This report aims to evaluate the potential of bacteriophage-based diagnostics – particularly genetically engineered reporter phages – as innovative tools for detecting mastitis

pathogens rapidly and specifically at the point of care. The primary objective is to assess the feasibility of incorporating bacteriophage systems into existing dairy workflows to provide fast, cost-effective, and accurate identification of the causative bacteria responsible for intramammary infections. Secondary objectives include exploring the mechanisms and advantages of phage-based diagnostics, analyzing their stability and multiplexing potential, and examining their broader applications in the dairy industry and food safety. By addressing these aims, this report seeks to demonstrate how bacteriophage diagnostics can bridge the current gap between disease detection and targeted treatment, thereby improving therapeutic precision, reducing antimicrobial misuse, and enhancing both herd health and food system sustainability.

Chapter 1: Background

Mastitis and the Need for Improved Diagnostics

Bovine mastitis is considered the most common disease leading to economic losses in the dairy industry, with global annual losses estimated to be up to $32 billion (USD) (1–3, 5). Economic losses are due to decreased milk production, diminished quality, discarded milk, premature culling, therapeutic expenses, and labor charges (1). The largest contributing factor to economic losses is decreased milk yields, which is estimated to account for up to 70% of the total economic losses (1, 2). Early, reliable, simple, and cost-effective diagnostics are essential to mitigate economic losses and treat infections effectively.

Despite advances in on-farm management and treatment, mastitis remains highly prevalent in North American dairy herds, underscoring the urgent need for better diagnostics. In Canada, for example, a study of 106 dairy farms reported a mean incidence rate of 23 clinical mastitis cases per 100 cow-years, with individual herds ranging from 0.7 to 97.4 cases per 100 cow-years (8). Meanwhile, in U.S. dairies, recent cohort data suggest an incidence rate around 24.4 cases of clinical mastitis per 100 cow-lactations, with older cows being particularly affected (9). Subclinical mastitis is even more widespread and insidious: globally, meta-analyses estimate its pooled prevalence near 42 percent, with North America among the highest-prevalence regions (10). The data highlights that clinical mastitis, while costly and obvious, is only the tip of the iceberg; subclinical infections silently persist, reducing herd health and productivity.

Beyond financial costs, mastitis imposes serious non-economic burdens on animal welfare, milk quality, and the environment. From an animal welfare perspective, mastitis causes inflammation, pain, and discomfort; inflamed udders may be swollen, hot, or sensitive, and infected cows often suffer systemic effects such as fever or reduced appetite (11, 12). These

welfare impacts are underrecognized but critically important. In terms of milk quality and safety, mastitic milk has elevated somatic cell counts (SCC), which correlate with bacterial load, and even in subclinical cases, the milk may harbor higher concentrations of pathogens while remaining in the bulk tank (12). From an environmental standpoint, repeated treatment of mastitis often involves antibiotic use, which leads to excretion of drug residues, antibiotic-resistant bacteria, and resistance genes into manure. When this manure is applied to fields, such residues may leach into soil or run off into water systems, contributing to pollution and resistance dissemination (13, 14). Furthermore, manure management studies suggest that antibiotic persistence in soils can be long-lived, meaning that the downstream ecological impacts of mastitis treatment extend far beyond the dairy barn (14).

Bovine mastitis is defined as inflammation of the mammary gland that can be caused by either physical trauma to the tissues or microorganism infection (1, 4). The most common cause of mastitis is an intramammary infection (IMI). The terms mastitis and IMI are generally used interchangeably, although no single diagnostic test can define both. A diagnosis of mastitis is generally based on measurement of the inflammatory response, whereas diagnosis of an IMI is based on identification of the infectious agent causing the inflammatory response. Mastitis is diagnosed by measuring inflammation indicators, and this diagnosis is generally used as an indirect method to identify cows with an IMI (4). Therefore, the diagnosis of an infectious cause for mastitis is a two-step process, with general diagnostic tests to identify mastitis conducted first followed by further diagnostic methods to identify the infectious organism (4, 7).

Currently, this second step of the diagnostic process for an IMI is not commonly performed, and the causative agent is often not diagnosed. Farms will opt to use systemic antibiotics as soon as possible after detection rather than determine the cause (2, 5). Besides the

economic impacts of mastitis on the dairy industry, this widespread antibiotic use has public health implications with concerns of adverse effects on humans who consume milk and meat products of treated animals, and the possible development of antibiotic resistance due to generalized usage (2, 6). Continuous exposure of bacteria to antibiotics can lead to selection and exchange of antibiotic-resistant genes in bacterial populations, leading to the continued selection and prevalence of antibiotic resistance populations in the environment. Resistance selection in bacteria due to constant exposure raises concerns to the possibility of this extensive antibiotic usage to treat mastitis leading to food-producing animals being reservoirs of resistance genes that disseminate the genes to other bacterial populations (2, 5). Several studies have shown an increased prevalence of resistant bacteria in commensal bacterial populations of antibiotic-treated dairy animals, as well as finding resistant food-borne pathogens in dairy farm soil (2). Mastitis is the primary reason for the usage of antimicrobials in dairy cows, with up to 80% of all doses being targeted at combating mastitis cases (1, 2). The absence of effective pathogen-specific diagnostics in mastitis cases perpetuates both economic and public health risks, highlighting the need for improved diagnostics to enable targeted intervention.

Current Diagnostics and Limitations

Due to the economic burden mastitis poses, there is a need for accurate detection and prevention of mastitis. On-farm screening tests are currently used for the initial diagnosis of mastitis, and they include somatic cell count (SCC), California mastitis test (CMT), and Surf field mastitis test (SFMT) (7). Somatic cell count is a measurement of the concentration of leukocytes per milliliter of milk and is measured per teat, commonly referred to as a quarter. In uninfected quarters, 80% of the somatic cells in a sample will be leukocytes, compared to 99% in

infected quarters (4). Somatic cell count is treated as a universal indicator for mastitis detection and determination of milk quality in individual quarters and bulk tanks. The CMT and SFMT are simple tests performed on site to provide an SCC estimation. The tests are easy to perform, reagents are cheap, and results are comparable to each other. The ease and reliability of the tests makes SCC the current most widely used biomarker test for bovine mastitis diagnosis (4, 7).

However, an SCC does not provide information on the cause of mastitis – infectious or otherwise – and is instead a general indicator of milk quality and disease detection. Identification of the causative agent is important in formulating an educated treatment plan as it allows for selective antibiotic usage, informed culling decisions, and improved control strategies to prevent spread of contagious agents (2, 5, 7). Antibiotic use is the main strategy to treat mastitis, but it can be an ineffective treatment method; different categories of pathogens require various management strategies, and without identifying the causative pathogen it is unknown if the chosen antibiotic will be effective (1). In some cases, antibiotics are unnecessary – Gram-negative bacterial infections tend to clear themselves quickly and do not need antibiotic treatment (3). The success of mastitis treatment is dependent upon the causative pathogen, so identification of the pathogen is critical for proper targeted therapy (1, 7).

The gold-standard for identification of an IMI pathogen is culture-based techniques, but the methods have high rates of false negative results with reported probabilities for a false negative result of 20-50% (2, 4, 7). Culturing takes a great deal of time (approximately 16-48 hours) and is labor-intensive, requiring trained personnel. Unless a farm has an on-site laboratory with trained personnel, samples must be shipped for testing. On-farm culture kits have been developed, and validation of the systems is ongoing, but the time to results is still high at 16-24

hours and the usage of the system by farmers still requires investment into dedicated facilities, equipment, and personnel training (2).

Molecular diagnostics – via amplification or sequencing – are much faster in comparison, taking less than 4 hours with increased sensitivity and specificity compared to culturing. The downsides are a significant cost increase, requiring a dedicated lab with expensive equipment, reagents, and trained personnel. See Table 1 for further comparison between bacterial culture and PCR-based techniques. PCR-based diagnostics also require sample pre-treatment, as the assay is sensitive to many components of milk. While various molecular assays detect multiple pathogens, this method does not distinguish between living and dead cells, making it uncertain whether an identified pathogen is the cause of disease or remnants of a previous infection or environmental contaminant (2, 7).

Table 1. Comparison of bacterial culture and PCR-based approaches

	Bacteriologic Culture	**PCR**
Time to result	24 h – 10 d	4 h
Cost	Low	4-5x culture cost
Live organism detection	Yes	Not necessarily
On-farm usage	Limited	No
User-friendly	Yes	No
Sensitivity **Identify disease as positive**	Limited; misses nearly 50% of what PCR identifies	High
Specificity **Identify true negatives**	Environmental contamination	Environmental contamination; detects non-viable bacteria
Shelf life	Approximately 3 weeks at 2-8°C	12 months at -30°C to -10°C
Equipment needs	Consumables and incubator	Consumables and PCR machine

Adapted information from Martins et al. (2019), Adkins et al. (2018), and Ashraf et al. (2018) (2, 4, 7)

The presence of a potential mastitis pathogen also may or may not be indicative of an IMI – bacteria could come from outside sources during sample collection such as contaminated teat skin or improperly sanitized equipment (4). Consequently, there are recommended thresholds to conclude a positive result for both culture and PCR based testing methods. For culture

techniques, the standard plate count (SPC) provides an estimate of the total bacterial load in the bulk tank, the laboratory pasteurized count gives an estimate of thermoduric bacteria, and the preliminary incubation count estimates the number of psychotropic bacteria (4). The recommended thresholds for SPC, laboratory pasteurized count, and preliminary incubation count are less than 5,000 CFU/mL, less than 100 CFU/mL, and less than 10,000 CFU/mL, respectively (2, 4). The results of a PCR assay are presented as a cycle threshold (Ct) value, with a lower Ct value indicative of a greater amount of the target genetic material being detected. The recommended threshold for a positive result via PCR assay is a Ct value of ≤37.0. (4)

Due to the extended time to results and need for trained laboratory analysis, the causative agent of mastitis is often not identified. Instead, a presumptive mastitis diagnosis determined by SCC will be immediately treated with systemic or intramammary antibiotics (2). Without an identified cause of infection, this antibiotic treatment may be wholly ineffective, leaving the infection improperly treated with poor outcomes for the animal and potentially contributing to antimicrobial resistance (AMR). This treatment can also leave residual antibiotics in milk. If residues are detected, that milk cannot enter the food chain, leaving product wasted (2, 6). The development and implementation of an effective, affordable, on-farm diagnostic that easily fits within the already established dairy workflow would increase the likelihood of the causative agent being identified and treated accordingly, resulting in better treatment plans and outcomes. Several emerging diagnostic platforms – including bionsensor-based assays, microfluidic systems, Loop-Mediated Isothermal Amplification (LAMP) amplification, Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) profiling, and rapid immunoassays – are also being explored to improve the speed and accuracy of mastitis detection (2, 4, 6, 7). However, many of these technologies still require specialized instrumentation, controlled

laboratory conditions, or substantial financial investment, limiting their suitability for routine on-farm use (2, 6, 7).

Chapter 2: Bacteriophage as a Diagnostic Assay

Bacteriophage Biology

Bacteriophages, commonly referred to as phages, are viruses that specifically target and infect bacterial cells. They are the most abundant biological entities on the planet and play a crucial role in regulating bacterial populations in both natural ecosystems and manmade environments (15). They are highly selective, with each bacteriophage targeting only a specific bacterial species with some being selective down to the strain – a trait that makes them ideal for diagnostic applications (15–17). In contrast to molecular techniques that detect genetic fragments or protein signatures, phages operate at the level of active infection, offering an indicator of live bacterial presence (17–19).

Phage infection begins when a virion binds to receptors on the surface of its target bacterial cell. The phage injects its genetic material into the host, and one of two lifecycles begins depending on the type of phage (15, 17). A lytic phage will start the lytic cycle, where the injected genetic material hijacks the bacterial cellular machinery to replicate phage genomes and synthesize new virion particles. This life cycle culminates in lysis of the host cell and release of progeny phages, which move on to the next bacterial cell to continue the infection cycle (15). A lysogenic phage will enter the lysogenic cycle, where the injected genetic material will become integrated into the bacterial chromosome forming what is called a prophage. The prophage replicates passively along with routine bacterial cell division, until certain stressful environmental conditions are met and the prophage will be excised from the bacterial chromosome to then enter the lytic cycle as previously described (15). This inherent biological amplification, where a single phage particle can yield hundreds of new virions, provides a natural signal boost that removes the need for external amplification (16, 17). Moreover, because phages

rely on metabolically active hosts to replicate, they offer an advantage over PCR. PCR diagnostics can detect genetic material from dead cells and lead to an inaccurate diagnosis of a previously cleared infection (18, 19). Phages, by comparison, will only infect and detect active host cells and does not risk detecting cell debris.

Phage diagnostics have demonstrated applicability in diverse settings including clinical medicine, environmental monitoring, and food safety, where they have been used to detect pathogens like *Listeria monocytogenes* and *Escherichia coli O157:H7* (17, 20, 21). Their potential for bovine mastitis diagnostics lies in their specificity, scalability, and adaptability to complex matrices such as milk.

Reporter Phages

To translate the infection event into a measurable output, researchers have developed reporter phages – bacteriophages engineered to carry genes encoding easily detectable markers. These genes can produce markers that are fluorescent, bioluminescent, or colorimetric (17). Upon infecting a suitable host, the phage's genome is replicated and expressed, including the inserted reporter gene, which produces a measurable signal change. A reporter phage producing a fluorescent protein will require an excitation step to cause the fluorophores to produce a measurable signal. A luminescent reporter phage results in a signal output of light and signal would only be visible in a dark room or with an instrument. Colorimetric reporter phages will produce a protein that will require exposure to a separate substrate to produce a visible color change (17, 18, 20). A fluorescent, bioluminescent, or colorimetric reporter phage approach creates a binary and possibly quantitative readout that directly corresponds to the presence of live, susceptible bacteria (17, 18, 20).

The selection of an appropriate reporter system is critical, particularly when working with milk samples. The opacity and natural fluorescence of milk can interfere with a fluorescent or bioluminescent readout (17, 21–23). Cases of clinical mastitis result in visibly abnormal milk including clots, flakes, and/or changes in color and consistency which can also cause signal interference with a reporter phage system (4). Although bioluminescent (e.g., luciferase) and fluorescent markers have proven useful in laboratory environments, they often require sensitive instruments and controlled conditions (17, 18). For field applications, colorimetric systems are more promising as they produce a signal that is visible to the naked eye. If the goal of the field test is a binary present/not present result, a colorimetric readout would therefore not require instrumentation. For example, phages engineered to express β-galactosidase can produce a visible color change in the presence of substrates like chlorophenol red-β-D-galactopyranoside (CPRG), yielding a red signal that can be visually observed without instrumentation (22, 24).

Developing an effective reporter phage involves several technical challenges. The modified genome must retain infectivity and ensure stable expression of the reporter gene without compromising the ability of the phage to propagate and increase signal strength (17, 19, 20). Additionally, genetic elements must be carefully selected to ensure high signal-to-noise ratios and limit cross-reactivity or inhibition (21, 25). There is also a low threshold detection level that is not due to signal output, but rather the low likelihood of phage randomly interacting with and infecting a cell in a given space when the bacterial concentration is low (21). Despite the complexities, proof-of-concept systems for a range of bacterial pathogens have been successfully demonstrated, featuring the viability of reporter phages in point-of-care diagnostics (17, 18, 21).

A reporter phage–based diagnostic also offers the advantage of being inherently low-cost compared to conventional laboratory methods. Unlike PCR, which requires thermal cyclers, fluorescent probes, trained personnel, and specialized laboratory infrastructure (2, 4, 7), reporter phages rely on biologically driven amplification and simple visual readouts. The phage performs both target recognition and signal generation within a single step, removing the need for enzymatic reagents, nucleic acid extraction kits, or precision-controlled instrumentation (17, 18, 20). Reporter phages can be produced at scale using inexpensive bacterial cultures, and once purified, the preparations can be lyophilized for room-temperature storage (22, 26, 27). Lyophilization eliminates the cost of cold-chain transport while preserving phage viability for extended periods, further reducing overall per-test expenses (22, 26, 27).

Additionally, the procedural simplicity of reporter phage assays helps minimize operational and implementation costs. A workflow that requires only sample addition, brief incubation, and visual inspection reduces hands-on labor and eliminates the need for specialized training or controlled laboratory environments (17, 20, 21). Because reporter phages generate a signal only when infecting viable target bacteria, they also reduce the need for confirmatory testing to distinguish true infections from environmental contaminants or non-viable genetic fragments – an issue common in PCR-based diagnostics (4, 17, 18). Collectively, these biological, logistical, and operational advantages make reporter phage diagnostics well-suited for on-farm use where financial, labor, and time constraints limit adoption of conventional testing technologies.

Biosafety and Regulatory Considerations

While reporter phages offer clear advantages for rapid and specific pathogen detection, their development and deployment must also consider biosafety and regulatory constraints. From a safety standpoint, bacteriophages are generally recognized as safe (GRAS) for human exposure and have a long history of use in food processing and environmental applications (26, 28–31). However, reporter phages introduce additional oversight considerations due to the presence of engineered genetic elements. Regulatory agencies increasingly require assurance that inserted reporter genes do not confer unintended traits, such as altered host range, enhanced environmental persistence, or the potential for horizontal gene transfer to microbial communities (17, 20, 25). These concerns are particularly relevant for diagnostic assays intended for on-farm use, where phages may encounter soil, wastewater, or manure.

Because reporter phages are genetically modified organisms, their use in veterinary diagnostics would fall under the regulatory authority of the United States Department of Agriculture (USDA) Center for Veterinary Biologics (CVB). CVB licensing requires demonstration that the phage construct is genetically stable, does not produce adverse effects in the target species, and does not present risks to the environment under conditions of intended use. Validation studies must also show consistent manufacturing quality, absence of adventitious agents, and well-characterized genomic modifications (32, 33). Furthermore, classification as a diagnostic reagent rather than a therapeutic biologic requires evidence that the phage does not replicate to levels posing environmental or animal safety concerns, and that its application results in minimal dissemination outside the reaction vessel or test matrix (32, 33).

In practice, these regulatory expectations mean that reporter phage diagnostics must be evaluated not only for analytical performance but also for containment, genetic stability, and

environmental compatibility. Strategies such as engineering non-replicating or replication-limited phage variants, incorporating kill-switch elements, or designing assays where phages remain confined within closed reaction vessels can mitigate biosafety concerns while simplifying the regulatory pathway (17, 20, 25). Collectively, addressing these biosafety and regulatory requirements early in assay development will be essential to achieving approval for field deployment and ensuring responsible use of engineered phages in agricultural environments.

Stability, Specificity, and Readout Efficiency

One of the most compelling advantages of phage-based diagnostics is the robustness of the reagent itself. Phages are inherently stable in a range of environmental conditions and can retain their infectivity across various sample types, including milk, blood, and water (15, 17, 21, 22, 26). Their shelf life can be extended through lyophilization, a process that desiccates the phage preparation into a powder that can be reconstituted with sample fluid. Lyophilized phages stored at room temperature have been shown to remain viable for months, making them particularly suitable for use in field conditions without refrigeration (22, 26, 27).

Phages also provide unparalleled specificity. While PCR can sometimes detect non-viable cells or experience cross-reactivity with closely related organisms, phages inherently discriminate based on viable, actively replicating targets (4, 17, 18). For example, a phage specific to *Staphylococcus aureus* will not infect or signal in the presence of *E. coli* or commensal skin flora. This fine-tuned specificity minimizes false positives and enhances confidence in results. Traditional culture-based methods, although regarded as the diagnostic gold standard, can also fail to detect certain pathogens due to factors such as low bacterial concentrations, slow-growing or fastidious organisms, or prior antibiotic treatment that

suppresses bacterial viability without full eradication (2, 5, 7). In such cases, pathogens may remain undetected even though infection persists, leading to false-negative culture results and misinformed treatment decisions (5). By contrast, phage-based assays directly target viable bacterial cells, maintaining diagnostic sensitivity even when pathogen numbers are low or when mixed microbial populations are present in complex samples such as milk (17, 18, 22).

Perhaps most importantly, phage-based systems can yield results within a remarkably short timeframe. Studies have shown that luminescent phages can produce a detectable signal within 10 to 30 minutes of exposure to a target organism at certain target concentrations (34). Colorimetric phages have produced detectable signals in 4 hours or less, making the result time comparable to current PCR methods (22, 24). In the context of mastitis diagnostics, this rapid time to results and on-site applicability allows for pathogen identification and treatment decisions to be made same day, significantly improving the timeliness of interventions and reducing reliance on empirical therapy.

The ease of interpretation and procedural simplicity further support field application. An ideal workflow would involve adding a milk sample to a pre-loaded reaction tube containing lyophilized phages and substrate, followed by visual inspection for a color change after a brief incubation. Such a test could be administered by farm staff with minimal training, offering a substantial shift from the complexity and delay associated with lab-based diagnostics.

As noted in Chapter 1, the presence of a potential mastitis pathogen does not inherently confirm an intramammary infection, underscoring the need for diagnostic platforms that meet rigorous performance standards (4, 7). For a phage-based assay to be viable, its signal output must align with established interpretive thresholds used in culture- and PCR-based diagnostics, ensuring that positive results reflect clinically meaningful pathogen loads (2, 4, 7, 32). Validation

of such an assay would need to incorporate performance metrics commonly applied to mastitis diagnostics, including high diagnostic sensitivity and diagnostic specificity to accurately distinguish infected from non-infected quarters (2, 4, 7, 32). Assay accuracy – agreement between the phage readout and reference methods – would similarly be essential, particularly across diverse matrices such as normal, subclinical, and clinically abnormal milk (2, 7, 32).

In addition, defining a clear limit of detection (LOD) is critical, as phage-bacterium interactions depend on sufficient target density to ensure infection and reporter expression (17, 21). Per USDA CVB guidance, a dilution-based study should determine the lowest analyte concentration that reliably produces a positive result (32). Finally, strong repeatability and reproducibility, demonstrated through consistent performance across operators, days, and environmental conditions, would be required to show that the assay can function reliably under on-farm conditions (2, 17, 33). Environmental or procedural robustness ("ruggedness") should also be tested – for example, varying incubation temperature or sample volume – to show that small deviations do not dramatically affect the readout (33). Achieving these validation benchmarks will depend on optimizing phage concentration, sample volume, incubation parameters, and readout timing to ensure consistent and comparable results across herds and pathogen profiles (17, 18, 22).

Optimizing the phage concentration, sample volume, incubation conditions, and readout timing will therefore be instrumental, not only to achieve the required regulatory performance but also to ensure the assay is robust, reliable, and usable under typical on-farm laboratory or field-like conditions.

Multiplex Assay Potential

While single-pathogen detection is useful, mastitis is often a polymicrobial condition involving multiple infectious agents. Therefore, an ideal diagnostic would be capable of identifying multiple pathogens simultaneously. Multiplexing in the context of phage diagnostics is a promising area of development.

One approach involves the use of multiple distinct phages, each genetically engineered to carry a different reporter gene. When exposed to a mixed bacterial population, each phage would produce a unique color or signal if its specific host is present. For example, red, green, and blue indicators could be used to signal the presence of *Streptococcus uberis*, *Escherichia coli*, and *Staphylococcus aureus*, respectively. An obvious potential problem with this structure would be the misinterpretation of signals due to interactions between color readouts, substrates and/or matrix. Alternatively, separate test vials or wells could each contain a single phage type with the same reporter gene but differing target specificity. A separated test structure would allow for modular testing without signal interference and be an easier assay style to add further target analytes to as developed.

Though conceptually straightforward, multiplexing poses challenges in design and interpretation. Signal overlap, substrate compatibility, time to signal, and host range interference are all technical hurdles that must be addressed (17, 21, 26). Based on the desired bacterial targets, specific bacteriophages to infect the targeted organisms need to be identified and sourced. Many phages have yet to be characterized, and some are more amenable to genetic manipulation than others (20, 21, 25, 35). Insertion of a reporter gene without affecting the ability of the phage to infect will be difficult with uncharacterized bacteriophages. Moreover, minimal data currently exists for multiplex phage systems, with currently available data focused

on utilization of bacteriophage receptor binding proteins (RBPs) rather than phages themselves (36, 37).

Receptor binding proteins are the phage structural components responsible for recognizing and attaching to specific bacterial surface receptors. Because RBPs determine host specificity but can be expressed independently of intact phage particles, they offer a modular and highly controllable approach to pathogen detection. In this strategy, purified or engineered RBPs are immobilized on biosensor surfaces, fluorescently labeled, or coupled to nanoparticles, allowing simultaneous detection of multiple bacterial targets within a single assay (36, 37). This approach has achieved high specificity and avoided cross-reactivity by leveraging the natural binding selectivity of each RBP (36, 37). Receptor binding protein-based platforms offer another viable option for diagnostic development, although more technically difficult to develop than a reporter bacteriophage.

Beyond the known hurdles and limited development of multiplex phage systems, the theoretical foundation suggests that multiplex phage diagnostics could be a robust assay for comprehensive mastitis screening. By reducing time to diagnosis and enabling targeted treatment of multiple pathogens in a single workflow, these systems hold the potential to redefine standard practice in herd health management.

Limitations and Mitigation Strategies

While reporter phages present clear advantages for on-farm, rapid, and live-cell detection, several practical limitations must be recognized and addressed for reliable field deployment. First, phage reagents are biological entities whose performance can vary with formulation, storage, and handling. Physical stresses (temperature, moisture, agitation, freeze–thaw) and

matrix effects from milk can reduce infectivity and signal output; careful formulation (buffers, excipients), lyophilization, or powder-based delivery significantly improves shelf life and eliminates cold-chain dependence, but these approaches require rigorous stability testing under expected field conditions to define shelf life and storage recommendations (22, 27, 38, 39).

A second major limitation is bacterial evolution and phage resistance. Bacteria can escape detection or infection through multiple mechanisms – modification or loss of phage receptors, production of extracellular matrix or biofilms that block access, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas) or other adaptive immunity systems, and abortive infection pathways – which may reduce assay sensitivity over time or in particular strains (17, 21, 40, 41). Importantly, the selective pressure imposed by routine diagnostic exposure is different from therapeutic exposure, but resistance can still arise, especially if diagnostics are used repeatedly on the same herd without monitoring. Strategies to mitigate resistance include using phage cocktails or libraries to broaden host coverage, periodically rotating or updating phage panels informed by local isolate surveillance, and engineering reporter constructs to target conserved receptors or to use receptor-binding proteins (RBPs) with expanded specificity (36, 37, 42). Combining phage detection with parallel approaches (e.g., culture or targeted PCR for confirmation in ambiguous cases) can also reduce the risk of missed infections due to emergent resistance (4, 17, 43).

Scale-up, manufacturing quality control (QC), and regulatory compliance present further constraints for commercial adoption. Phage production that is straightforward at bench scale does not always translate directly to large bioreactors: upstream host–phage dynamics, multiplicity of infection (MOI), nutrient supply, and downstream purification (removal of host debris, endotoxin, and adventitious agents) must be optimized for consistency and yield (44, 45).

Meeting Good Manufacturing Practice (GMP) or diagnostic-grade QC standards requires validated processes, lot release testing (titer, infectivity, purity, genomic sequencing for stability), and documentation — all of which increase manufacturing cost and complexity, and must be planned early in assay development (32, 33, 46, 47). Regulatory pathways are still evolving for genetically modified reporter phages used as diagnostics; engagement with regulators (USDA CVB in the U.S.) to agree on containment, environmental risk assessment, and product classification (diagnostic reagent vs biologic) will streamline approval and field use (32, 46).

Diagnostic design and biosafety also shape limitations and mitigations. Reporter phages that are replication-competent could theoretically shed into the environment during on-farm use; this risk can be minimized by: 1. designing assays as closed, single-use cartridges where phages remain contained; 2. using replication-limited or non-propagating reporter constructs (e.g., plasmid-encoded reporters delivered by transducing particles or conditional replication systems); and 3. implementing clear disposal procedures in the kit instructions (17, 20, 48). These design choices reduce environmental release risk but may increase per-test reagent complexity or reduce signal amplification, so trade-offs must be evaluated during development.

Finally, routine maintenance of phage diagnostic systems – including lot tracking, stability monitoring, and local isolate surveillance – is necessary for long-term effectiveness. Practical measures include standardized QC release criteria (titer/infectivity threshold, absence of contaminants, genomic integrity checks), accelerated and real-time stability studies to set expiration dating, and an ongoing program to collect local bacterial isolates and test phage coverage (GMP/QC frameworks and continuous production models can help maintain consistency and lower per-unit cost at scale) (47, 49). Where resistance or coverage gaps are

identified, developers should be prepared to refresh phage panels or supplement diagnostics with RBPs or immuno-based probes that target conserved antigens.

To mitigate these limitations for on-farm mastitis testing, developers should: 1. prioritize robust formulation (lyophilized or stabilized powders) with defined storage conditions and validated shelf life; 2. use phage cocktails and RBP-based redundancy to reduce single-point failure from resistance; 3. design closed-cartridge assays or replication-limited reporters to minimize environmental release; 4. adopt GMP/QC-style lot release testing (titer, purity, genomic checks) and plan manufacturing scale-up early; and 5. implement ongoing surveillance and an update pathway to refresh phage panels when escape variants appear. Together these measures preserve the low cost, speed, and live-cell specificity advantages of reporter phages while addressing the practical, regulatory, and biological challenges to sustained field use (17, 22, 27, 38, 44, 46).

Chapter 3: Future Applications of Bacteriophage Diagnostics

Dairy Industry Applications

While the primary focus of this report is the use of bacteriophages for the diagnosis of bovine mastitis, the broader dairy industry presents multiple additional opportunities for the implementation of phage-based diagnostic tools. Infectious diseases such as metritis, lameness-associated foot rot, and respiratory conditions in dairy cattle also benefit from early and accurate diagnosis. These diseases, like mastitis, are commonly treated with antimicrobials, often without prior pathogen identification (11, 50, 51). By enabling rapid on-site detection, phage diagnostics could enhance disease management across a range of conditions, promoting more targeted antimicrobial use and improve outcomes (11).

In terms of practical implementation, phage-based assays could be seamlessly integrated into the existing dairy workflow. Lyophilized phage reagents can be distributed in single-use kits and stored without refrigeration, making them accessible even in rural or low-resource settings. A colorimetric or other visual signal system would allow for straightforward result interpretation by farm personnel without requiring specialized training. Such diagnostics could be used routinely as part of health monitoring protocols, for instance during monthly herd checks, during forestripping, or before dry-off periods, to guide selective dry cow therapy (4, 52).

The potential utility also extends to early lactation, a time when cows are particularly susceptible to infection (53). Detecting subclinical infections at this stage could prevent the establishment of chronic disease and reduce the need for later interventions (1, 4). As regulatory agencies and industry groups move toward stricter antimicrobial policies, the ability to base treatment decisions on confirmed diagnoses rather than presumptive symptoms will become an increasingly valuable asset to producers (2, 5, 6).

In addition to diagnosing and treating infections in the herd, bacteriophage-based interventions are increasingly being explored for the control of biofilms on dairy equipment and within milking systems. Biofilms formed by pathogens such as *Staphylococcus aureus*, *Streptococcus uberis* and other common mastitis-associated bacteria adhere to surfaces such as teat cups, hoses, bulk tanks, and stainless-steel pipelines, creating persistent reservoirs of contamination that resist conventional cleaning and sanitation (28, 54, 55). Studies have demonstrated that phages can significantly reduce biofilms on hard surfaces while retaining full activity in the presence of detergents (56), suggesting strong field-applicability for sanitation in dairy settings. Broader reviews of phage use in dairy environments confirm the potential for phages to act as disinfectants or adjuncts in sanitation programs, noting successful reductions in *E. coli* biofilms on food-processing materials such as stainless steel and high-density polyethylene (28).

Commercially, phage products such as those offered by PhageGuard are already marketed as processing aids in dairy operations, offering non-chemical and residue-free alternatives for surface decontamination (57). Integrating phage-based biofilm control into routine equipment hygiene would complement diagnostic advances and therapeutic interventions, offering a holistic approach to infection prevention, equipment sanitation, and antimicrobial management in dairy production.

Broader Food Safety Applications

Beyond their applications in animal diagnostics, bacteriophages are increasingly gaining recognition in the food industry as powerful tools for enhancing microbial safety. Their ability to selectively target and kill bacterial pathogens without disrupting beneficial microbes or altering

product quality has led to their adoption in various stages of food processing (28–31). One of the most well-established current uses of phages is antimicrobial washes applied to raw food products, particularly fresh produce and meats (30, 31). Phage-based washes are designed to reduce or eliminate harmful bacteria such as *Listeria monocytogenes*, *Salmonella spp.*, and *Escherichia coli O157:H7* from food surfaces before packaging and distribution (30).

In the United States, several phage-based products have received approval from the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) for direct application on food products. For example, Listex P100, a phage preparation targeting *L. monocytogenes*, is approved for use on ready-to-eat meats, cheeses, and smoked fish. When applied via spray or immersion, phage solutions reduce surface contamination without altering taste, texture, or nutritional content (26, 58). Studies have demonstrated that phage washes can significantly reduce bacterial loads in treated food products, contributing to extended shelf life and improved consumer safety (26). Similar approaches have been implemented in meat processing, where bacteriophage-based hide washes are used as an antimicrobial intervention to reduce *Escherichia coli* O157:H7 and *Salmonella* contamination on cattle hides prior to carcass dressing (26, 59). Commercial formulations such as EcoShield™ (Intralytix Inc.) and PhageGuard E (Micreos Food Safety) have shown measurable efficacy in lowering pathogen prevalence during beef processing, providing a residue-free and environmentally safe alternative to traditional chemical sanitizers (30, 31, 59). Phage-based applications underscore the growing regulatory and industrial confidence in bacteriophage technologies as safe and effective tools for controlling foodborne pathogens across multiple processing stages.

The adoption of phages in food processing not only provides an effective biocontrol measure but also enhances public confidence in food safety. Importantly, phages are classified as

Generally Recognized As Safe (GRAS) by regulatory agencies, supporting their use as non-chemical, residue-free antimicrobials. Their targeted mechanism of action minimizes disruption to food microbiomes and avoids the broad-spectrum ecological consequences often associated with conventional sanitizers or antibiotics. Unlike harsh chemical treatments, phages are non-corrosive to equipment, improving longevity of equipment lifespan, a further benefit to producers (29–31, 59).

Building upon this foundation, the next step involves leveraging phages for microbial detection in the food supply chain. Phage-based biosensors and diagnostic strips can be developed to rapidly detect bacterial contaminants at critical control points. Diagnostics of this type could be deployed in dairy plants to screen raw milk before pasteurization, in food packaging facilities to monitor surface hygiene, or even in retail settings to assess spoilage. Another promising innovation is the use of bacteriophage in the development of "active packaging" – packaging in which active ingredients have been added to improve performance (60). The use of phages as antimicrobial agents added to produce active packaging has been successfully explored with various packaging techniques utilized (60, 61). Modifying this concept and utilizing reporter phages in packaging to produce a visible color change in the presence of specific pathogens could provide a visible indicator of contamination or spoilage to consumers and handlers.

The transition from phage treatment to phage detection is a logical extension of their proven effectiveness and safety in food systems. As the dairy and broader food industries adopt more comprehensive approaches to pathogen control and traceability, bacteriophage diagnostics offer a natural complement to existing biocontrol strategies. Their integration could facilitate a

more proactive model of food safety—one that emphasizes early detection, risk reduction, and consumer assurance from farm to fork.

Clinical and Environmental Diagnostic Uses

The principles underpinning phage diagnostics are not limited to agricultural or food settings. In human and veterinary medicine, there is growing interest in using phage assays to detect infectious agents in various biological matrices. For instance, blood, urine, and sputum have all been successfully tested for the presence of bacterial pathogens using reporter phages in laboratory settings (17, 21). The matrices represent common sites of infection and are often difficult to analyze rapidly using conventional diagnostics (21).

The low cost and ease of use of phage-based systems make them particularly suitable for deployment in low-resource clinical environments. In veterinary practice, mobile phage-based diagnostic kits could be brought directly to farms, zoos, or wildlife conservation areas, providing actionable results during a single visit. In human medicine, similar kits could be used in field hospitals, rural clinics, or outbreak response units, offering timely information for both patient management and epidemiological tracking.

Environmental monitoring also stands to benefit from phage-based tools. Water testing for bacterial contamination, for example, could be streamlined using phage sensors that detect enteric pathogens like *Salmonella* or *Shigella* (62). Likewise, agricultural runoff and manure lagoons – potential sources of zoonotic pathogen transmission – could be monitored more effectively with rapid phage-based tests (2). The applications highlight the versatility of phages as biological tools capable of addressing public health challenges at the interface of agriculture, ecology, and medicine.

Bacteriophage Therapy

Bacteriophages have been investigated as therapeutic agents for nearly a century due to their ability to selectively target and lyse pathogenic bacteria while sparing beneficial microbiota (63–65). In the context of bovine mastitis, phage preparations can be applied topically to the teat or infused into the mammary gland to treat infections caused by antibiotic-resistant or recurrent pathogens (28, 64, 65). One strategy involves the development of phage cocktails, which combine multiple phages targeting the most common mastitis-causing bacteria, such as *Staphylococcus aureus* and *Escherichia coli*. The cocktails are designed to broaden host range and reduce the likelihood of phage resistance, and studies have demonstrated that such formulations can significantly reduce bacterial load and inflammatory responses (28, 64, 65)

Phage therapy has also been investigated in combination with other treatments, including antimicrobial peptides, immune modulators, and conventional antibiotics. Such combinations may enhance therapeutic efficacy, prevent relapse, and reduce the emergence of phage resistance (28, 63). Despite promising results, phage therapy faces regulatory and technical challenges. Production scalability, host immune responses, and potential development of phage resistance remain key considerations, and clinical adoption is limited by regulatory frameworks that are only beginning to accommodate phage-based products in the United States and Europe (28, 63, 64).

While promising, phage therapy still faces significant regulatory and technical challenges. Nonetheless, the potential to combine phage diagnostics and therapeutics into a unified “detect-and-treat” platform is particularly attractive. Such systems could allow rapid detection of mastitis pathogens followed by immediate targeted treatment, potentially reducing reliance on broad-spectrum antibiotics and mitigating the spread of antimicrobial resistance (65).

Once phage therapy systems are built and established for mastitis detection and treatment, a regulatory and technical development pathway will be opened for further bacteriophage usage and treatment for other common dairy cattle diseases such as metritis, lameness-associated foot rot, and respiratory conditions (11, 50, 51).

Conclusion

The persistent burden of bovine mastitis on the dairy industry underlines the need for rapid, accurate, and practical diagnostic solutions (1, 2). Current diagnostic methods, though well-established in laboratory contexts, fall short in terms of speed, affordability, and ease of use at the point of care (4, 7). As a result, producers frequently resort to broad-spectrum antibiotic treatment without identifying the causative pathogen, a practice that contributes to both treatment inefficiencies and the acceleration of antimicrobial resistance (2, 5). In this context, bacteriophage-based diagnostics emerge as a highly promising alternative.

Bacteriophages possess several inherent advantages that align well with the demands of field diagnostics. Their specificity allows for accurate pathogen detection even in complex sample matrices like milk, while their ability to replicate and self-amplify enables strong signal output with minimal external input (15–17). Reporter phage systems have further enhanced this capability by linking suspected infections to easily measurable outputs, including colorimetric changes visible to the naked eye (17). Such features open the door to on-farm diagnostics that can inform treatment decisions in real time, potentially transforming the standard approach to mastitis management.

Beyond mastitis, phage diagnostics have broad applicability across veterinary medicine, environmental monitoring, and public health. The dairy industry stands to benefit not only through disease management but also through enhanced food safety. Bacteriophages are already in commercial use as antimicrobial agents in produce and meat processing, demonstrating both efficacy and regulatory acceptance (30, 31). Extending this application to microbial detection is a natural and useful scientific progression. Emerging technologies such as phage-embedded

biosensors and active packaging further illustrate the untapped potential of this biological toolset (60).

As the global agriculture and food sectors move toward more sustainable and precise practices, diagnostic platforms that are fast, reliable, and field-deployable will be essential. Bacteriophage diagnostics, with their combination of specificity, scalability, and compatibility with low-resource settings, offer a compelling solution. However, further investment in research and development – particularly in multiplexing strategies, field validation, and regulatory pathways – will be critical to bringing phage tests to fruition. With continued innovation and cross-disciplinary collaboration, phage diagnostics may soon become a cornerstone of modern animal health and food safety strategies.

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