Design Brief

Bo-Find: A rapid, user-friendly diagnostic tool for bovine respiratory diseases*

Gabe Anoos, Mercy Awosoga, Scarlett Davis ▫ Catholic Central High School, Lethbridge, AB

Jovin Bach, Ry Baker, Jayabhishek Chaudhary, Dana Omar, Carlos Saunders ▫ Chinook High School, Lethbridge, AB

Elyse Burgess, Nicole Burgess, Nora Laird, Marie Metz, Khushi Patel, Nour Salmi, Maya Walton ▫ Lethbridge Collegiate Institute, Lethbridge, AB

Ian Benkoczi, Arman Bidarian, Ziqiao Chen, Mitchell Conrad, Maliha Kabir, Selina Sun, Keitaro Tatsuno, Samantha Walker, Jerry Wang, Zitong Wu, Bobby Zhao ▫ Winston Churchill High School, Lethbridge, AB

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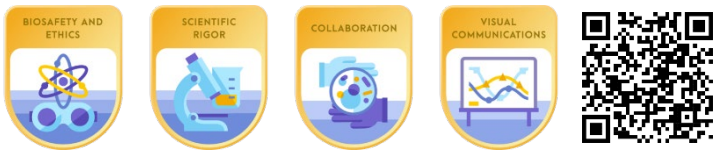
Alberta, Canada is home to more than 4.5 million heads of cattle and is the largest cattle producing province. Bovine respiratory disease (BRD) has a significant economic impact on cattle production, as approximately 15% of cattle in North America are treated each year. BRD is caused by several different bacterial and viral pathogens. Four of the most common bacterial pathogens (Mannheimia haemolytica, Pasteurella multocida, Histophilus somni, and Mycoplasma bovis). Mistreatment of infected cattle can lead to an increase in antimicrobial resistance genes (ARGs) and loss of antibiotic efficacy. Currently, only traditional bacterial culture and PCR analysis of the pathogens can provide accurate bacterial diagnosis; however, results can take up to a week. In order to quickly and successfully diagnose BRD pathogens in the field, we propose the use of a recombinase polymerase amplification (RPA)-based tool that can reliably give test results in under an hour and without laborious sample preparation or analysis.

Keywords: Cattle, respiratory, recombinase polymerase amplification, detection



The Alberta cattle industry accounts for over $470 million of Alberta's GDP (Stats Can, 2017). Since it is a huge economic driver for the province, maintaining healthy agricultural practices is extremely important. Bovine respiratory disease (BRD) is one of the most common diseases among cattle. It is mostly prevalent among young calves and is caused by a mix of pathogens, environment, and management factors. The four main bacterial pathogens are *Mannheimia haemolytica, Histophilus somni, Pasteurella multocida, and Mycoplasma bovis* (Confer, 2009). These bacteria are transmitted by direct contact, use of contaminated equipment, and stress that weakens the immune system as a result of transport or a change in diet. Symptoms include weight loss and dehydration in calves; in cattle: rapid breath, coughing, and,

* *The authors were mentored by Laura Keffer-Wilkes from University of Lethbridge. Please direct correspondence to: kefferwilkesl@uleth.ca.*

in severe cases, pneumonia are all indicators. BRD is currently treated by giving the cattle antibiotics with the hope that the infection will pass (Taylor et al., 2010). This affects farmers as it can cause cattle and economic losses.

Current Diagnostic and Treatment Solutions for BRD

Currently, no vaccines against these BRD infections are used by producers. Treatment of sick cattle is usually through mass antibiotic administration through feed or water. Calves that are at high risk for BRD are metaphylactically treated with antibiotics (Taylor et al., 2010). However, mass antimicrobial administration represents major antimicrobial consumption and does not always result in decreased cattle mortality (Baptiste & Kyvsgaard, 2017). Unnecessary blanket treatment of animals can also lead to the increased spread of antimicrobial resistance genes and the decrease of antibiotic efficacy (Whiteley et al., 1992).

Clinical evaluations, which include highlighting symptoms of BRD, are the first step in diagnosing BRD. Veterinarians follow various multiple scoring systems, although research has pointed out a lack of consistency within these systems. Some protocols also rely on the experience of the person carrying out the examinations, which can be subjective and create inconsistencies (Kamel et al., 2024). Detecting fever in cattle is another diagnostic strategy for diagnosing BRD, and fever serves as a sensitive marker for detecting the disease. However, fever in cattle could be caused by various factors and not just BRD. Diagnosing BRD solely in the presence of fever can be challenging when other signs are not present. Behavioral assessments serve as an effective substitute for detecting BRD in the absence of on-farm scoring systems, although visual behavioral changes associated with BRD have been largely unresearched. Changes in behavior associated with BRD include a change in alertness, feeding patterns, energy levels, and boredom (Kamel et al., 2024).

The diagnosis of BRD is based on observation of clinical and behavioral signs, but these techniques can be subjective, time-consuming, and labor intensive. This process of diagnosis also requires proper training and lacks sensitivity and specificity. BRD is also found to be diagnosed too late, when the animal already has lesions. Automated registration systems are a technological diagnostic tool that does not require human presence and can detect changes in cattle that may be undetectable by visual assessment. These technological systems monitor physical behavior, feeding behavior, and spatial (movement) behavior. These technological tools use a radiofrequency identification system (RFID) to transmit information and identify which cattle display behavioral changes (Puig et al., 2022).

Accelerometers can be used to detect movement behaviors in cattle. Decrease in activity due to BRD can be confused with other problems in cattle, such as lameness or locomotive issues. The other issue with these physical behavior diagnostic methods also includes the high cost of these sensors and poor attachment to the animal (Puig et al., 2022).

Feeding behavior systems can monitor when the animal is at the feeder or drinker, measure daily feed/water intake, and the time these animals eat. Regarding drinking behavior, the results do not provide decisive information, so it is not a good variable for detecting BRD. It can also be noted that sick and healthy calves have no differences in drinking behavior. Spatial systems, using GPS, detect movement of the cattle, although GPS readings are not precise enough to distinguish the cattle doing specific activities, such as going to the feeder (Puig et al., 2022).

When considering BRD, antibiotics and vaccines are the most effective course of action to maintain the health of young calves and the herd. Some common pathogens that cause BRD include *Mannheimia haemolytica, Pasteurella multocida,* and *Histophilus somni*. These pathogens cannot be reliably handled with vaccination, leading to the usage of antimicrobials before incidence of BRD (Klima et al., 2014). The usage of antimicrobials acts as a management measure to reduce the prevalence of BRD in large cattle populations. Though this technique is currently proficient in preventing contraction, it raises the major risk of spreading antimicrobial resistance

genes in bacterial pathogens. If this technique continues to occur at such a high rate, it will ultimately be rendered useless as bacterial pathogens adapt to overcome it. Alternatively, antibiotics can be used once symptoms are detected. Drugs such as florfenicol and tildipirosin are effective in treating bovine pneumonia, a characteristic of BRD (Tomazi et al., 2023). Early onset symptoms such as lethargy, shallow breathing, and fever can initially be hard to detect. Often, these symptoms do not appear until after the pathogen has caused BRD. This delay between contraction and treatment can be problematic and result in reduced efficacy. Treatment of BRD is also very expensive. This condition is economically devastating to the farming industry, causing an annual loss of $800-900 million dollars to the American feedlot industry. This cost includes lost money due to cow mortality as well as high treatment and prevention costs (Blakebrough-Hall et al., 2020).

To prevent the spread of antimicrobial resistance genes, more accurate testing can be completed to identify the specific pathogen causing BRD in the animal. At present, this is done through traditional microbial culturing and PCR. Microbial culturing is relatively cheap, provides evidence of a live pathogen, and can give some indication of antimicrobial susceptibility. However, culturing BRD pathogens is rather time intensive and can take more than five days for results. PCR detection can be done using nasal swabs and isolated pathogen DNA. Therefore, no live microorganisms are required, it is highly sensitive, can be quantifiable and completed in under 24 hours. However, it does not detect live pathogens, is quite expensive and can give false positives. Antibody detection, such as ELISA, tests can also be completed, where vets look for BRD-specific antibodies in blood serum. These tests can take up to a week to complete and are unable to differentiate between vaccine induced or natural infection antibodies. Additionally, antibodies may perist in the serum long after the disease is resolved. Culture-enriched Direct MALDI-TOF can be completed on samples in 1 - 3 days but obviously requires a MALDI-TOF and technicians familiar with the technique. Similarly, nanosequencing of DNA samples could give results in 1 - 2 days but is expensive and needs method expertise (Pardon & Buczinski, 2020).

Alternative Diagnostic Techniques

Since the diagnosis of BRD is important for the proper treatment of the animals, new technologies have been tested to hopefully improve the efficiency of detection. Two such techniques are Loop-Mediated Isothermal Amplification (LAMP) and Recombinase Polymerase Amplification (RPA). Both techniques do not require the expensive equipment needed for PCR or qPCR.

Loop-Mediated Isothermal Amplification

LAMP is a method of DNA sequence amplification, established in 2000 by Notomi et al., as a faster, simpler, and more cost-effective alternative to the PCR method (Notomi et al., 2000). LAMP is used primarily for diagnostics and disease detection, rapidly detecting and identifying various pathogens in both field and clinical settings (Wong et al., 2018). LAMP does not require expensive thermal cycling equipment and can amplify DNA at a constant temperature, usually around 60 to 70°C (Soroka et al., 2021). This method of DNA amplification is quite rapid, able to amplify target sequences 10^9 times in under an hour (Notomi et al., 2000). LAMP's high level of specificity, due to its use of around 5 primers which recognize 6-8 distinct target regions, in conjunction with its rapidity, makes LAMP well-suited for use in field and point-of-care settings (Notomi et al., 2000).

Recombinase Polymerase Amplification

RPA is an isothermal DNA amplification technique (Daher et al., 2016). It produces an amplicon defined by the annealing of two opposing oligonucleotide primers (Figure 1). RPA functions at a temperature between 37 and 42°Cand requires enzymatic activity to power amplification. RPA occurs when the recombinase protein UvsX from T4 bacteriophage binds to primers, creating recombinase-primer complexes with primers that scan DNA for complementary sequences (Tan et al., 2022). The primers are inserted at

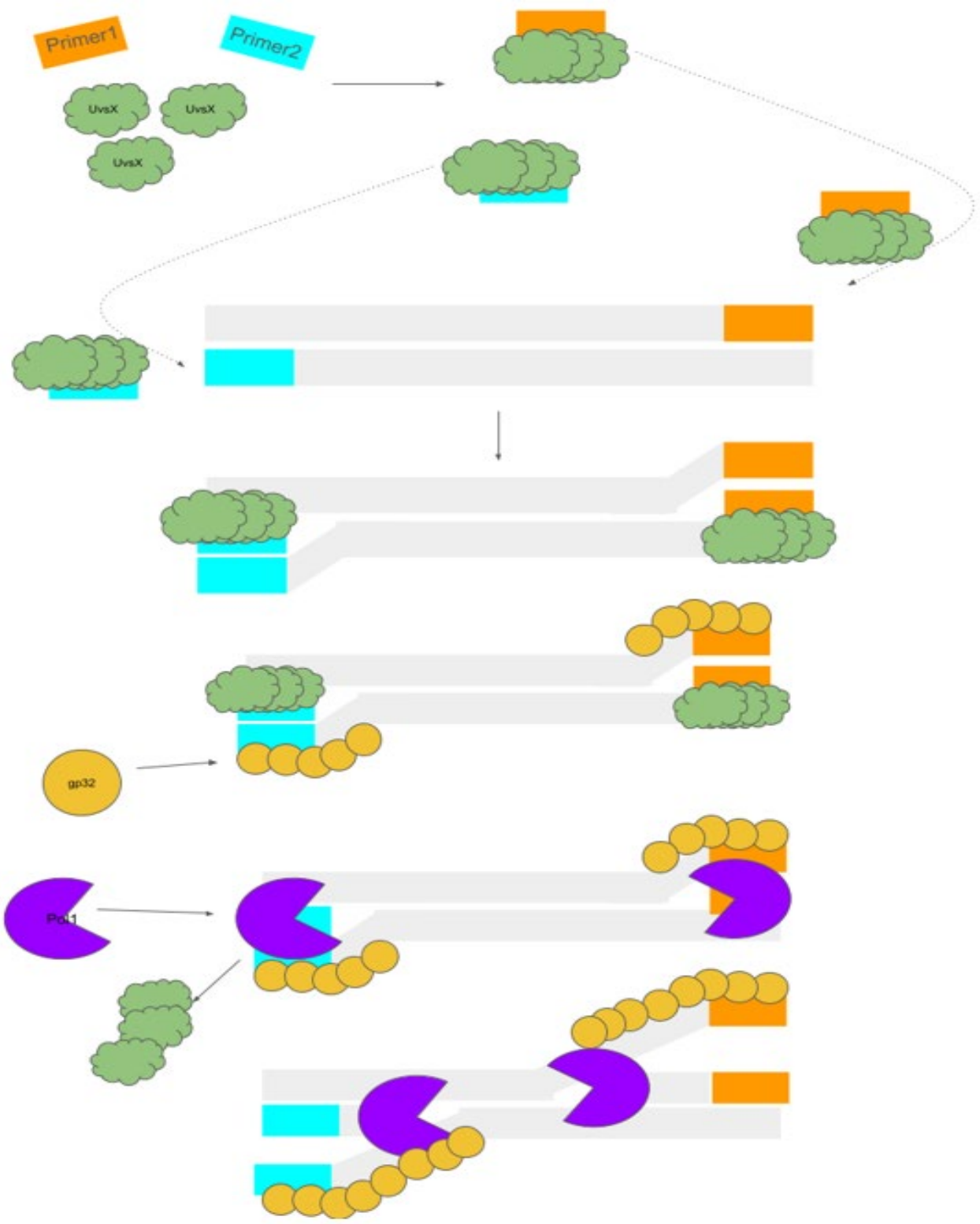


Figure 1. Schematic representation of recombinase polymerase amplification of DNA. DNA primers combine with recombinase proteins (UvsX). The complex scans the target DNA for homologous regions and displaces the DNA strand. Single strand binding proteins (gp32) binds to the single strand DNA. Recombinase leaves and DNA polymerase (PolI) recognizes the 3' end of the primer and starts elongation.

the target site, allowing them to anneal to the complementary DNA strand. ingle-stranded binding proteins (T4 gp32) then stabilize the displaced DNA chain, preventing the ejection of the annealed primer. The recombinase disassembles, leaving the 3'-end of the primers accessible to a strand-displacing DNA polymerase (the large fragment of Bacillus subtilis Pol I) that elongates the primer in the presence of dNTPs. The repetition of this process achieves amplification (Daher et al., 2016). RPA has been used with various detection strategies and is frequently used to monitor pathogens. It is an affordable, simple, and fast method for the identification of pathogens (Sumit Jangra et al., 2021; Magrina Lobato & O'Sullivan, 2018).

Systems Level

Our project aims to overcome several drawbacks to the current diagnostic methods. First, our device will be easy to use on-site at farms. We envision a hand-held device that vets or farmers can use (Figure 2). Second, sample preparation should be minimal. Current methods require extensive culturing and DNA preparation prior to pathogen identification or detection. Thirdly, our device will use a simple colour-change to give users a yes/no readout for infection. Finally, all our tests will be able to be carried out within one hour, speeding up the detection process.

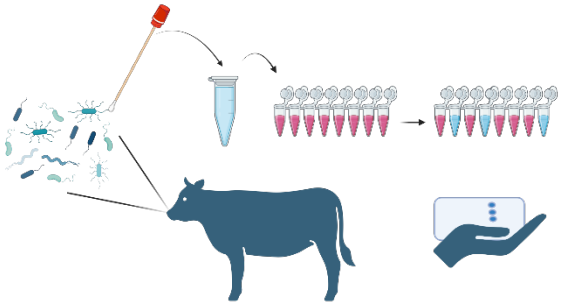


Figure 2. Bo-Find will incorporate on-site nasal swabs with recombinase-polymerase amplification technology to get rapid diagnostic results.

Device Level

Bo-Find would require vets or farmers to take a deep nasopharyngeal swab of the cattle. These swabs have been validated as a collection method for BRD testing (Crosby et al., 2022). DNA from the collected samples is typically isolated using a commercial kit, such as DNeasy Blood & Tissue Kit or QIAamp PowerFecal DNA Kit, both from Qiagen (Conrad et al., 2020; Reuter et al., 2020). However, we propose that may be unnecessary and a simple heat treatment step has been shown to be sufficient for sample preparation (Sumit Jangra et al., 2021). In our design, nasopharyngeal swabs would be added to a tube with a prepared extraction buffer solution. This tube would then be heated to 100°C for 10 minutes (Figure 3). The sample would then be transferred to the detection tubes. Each detection tube contains a different set of primers (Table 1 in Parts section) for pathogen detection. These primer sets were validated previously and have been shown to work with RPA detection (Conrad et al., 2020). As a proof-of-concept, we will begin our detection testing using purified plasmids from our *E. coli* library (Table 1). The RPA reaction will be allowed to start upon addition of the prepared sample DNA mixture, then incubated at 37°C for 30 minutes.

RPA is one of many new isothermal DNA amplification methods that have been developed in the last two decades (Magrina Lobato and O'Sullivan, 2018). RPA has many advantages including its high sensitivity, extremely rapid amplification, and simplified protocol. Briefly, recombinase proteins combine with DNA primers to form complexes, which together scan DNA for homologous sequences. The strand-displacement activity of the recombinase proteins allows for the insertion of the primers at their cognate sites (Figure 1). Single-stranded DNA binding proteins stabilize the displaced DNA strand and the recombinase disassemble leaving the 3'-end of the primers accessible to DNA polymerase, which can then in turn elongate the primer. RPA has been commercialized by TwistDx (Cambridge, UK) and will be used

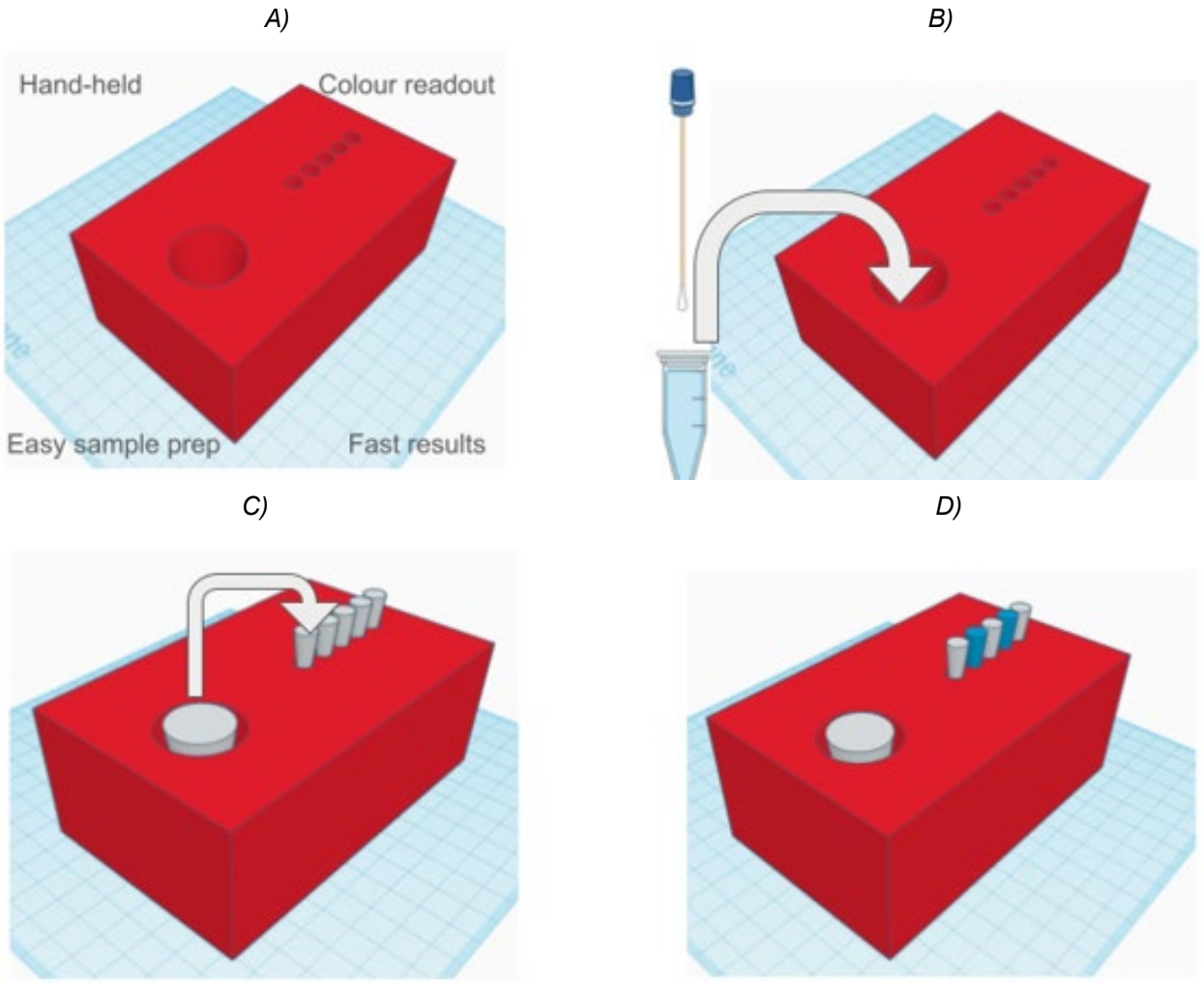


Figure 3. Hand-held temperature unit for sample incubation and detection (A). Nasal swab samples are first heat treated (B). Heat-treated nasal swab samples are loaded into individual detection tubes (C). Each tube contains a different primer pair for strain detection. A colour change indicates a positive result (D).

as a proof-of-concept for this project.

Finally, the amplified DNA will be detected through a simple colour change due to hydroxynaphthol blue (HNB). This chemical is a metal-sensing dye that changes colour in the presence (or absence) or magnesium ions. During DNA amplification (PCR, LAMP, RPA, etc), DNA synthesis generates pyrophosphate (PPi) as a byproduct. PPi in turn binds to Mg2+ ions and precipitates out of solution as $Mg_2P_2O_7$ (Figure 4). As magnesium is pulled out of solution, it can no longer interact with HNB and the colour changes from violet to sky blue. While the colour change can be easily observed with the naked eye, we anticipate creating a colour sensor that will be

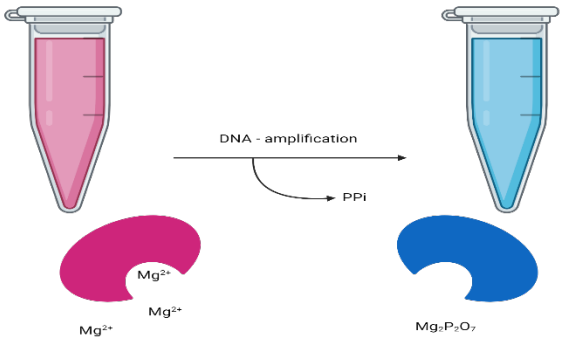


Figure 4. During DNA amplification, magnesium ions react with the pyrophosphate and precipitate. The depletion of magnesium ions in solution results in the colour change of hydroxynaphthol blue.

incorporated into our hand-held device. Ideally, the sensor would have a colour detection threshold and when this threshold is reached, it will send a message to the user's phone.

Parts Level

Genes for pathogen identification were chosen based on their specificity and uniqueness for that pathogen strain. Reference sequences for each species are available through GenBank. Proof-of-concept gene sequences are available through the iGEM parts registry.

Table 1. Primer pairs for detection of BRD pathogens and E. coli library plasmids.

Target	Gene	Fwd Primer	Rev Primer
Histophilus somni GenBank: CP000947.1	Hs_0116	CGTTTAATCCCATTG CGATCATTCCCCATT	ATACTATTGCATT CGGCGATTTTTC CGCTT
Mannheimia haemolytica A1 and A6 GenBank: NC_021082.1	*NmaA*	TCAAAATGGCTCCC TTAGTTGAGGGCTT TA	AGTGGTTGCTGT ATCGCCATGAAC AAAAAT'
Mycoplasma bovis GenBank: AF003959.1	*UvrC*	ATGGTCCTTTTCCTT CTGGTTATGGAGCT A	TGGCTGCTTGAT GCATTTTGTTAGT TAGTT
Pasteurella multocida GenBank: FJ986389.1	*Kmt1*	GAACCGATTGCCGC GAAATTGAGTTTTAT G	CCAACAAAACTG TGCTTTTCTTTGC CACAA
E. coli DH5α BBa_K1033933	*Aspink*	CAAAACCACGATTG AAGGCACGGTCAAC GGTCAC	GAACGCCGTCCA CTTCATAGACGA TTTCCGTAGCC
E. coli DH5α BBa_I20270	*Gfp*	GTCCCAATTCTTGTT GAATTAGATGGTGA TGTTAATGG	GGGCAGATTGTG TGGACAGGTAAT GGTTGTCTGG

Safety

Environmental, biological, and ethical safety are essential components of any research project. Investigation into proper disposal methods of prepared components is still required to prevent environmental contamination of pathogen DNA. While Oma et. al. (2018) showed the potential of specific BRD viral RNA spreading to other herds through short-lived human nasal mucosa carriage or 24-hour contamination of other fomites, however, a few BRD pathogens are known to be transmissible to humans.

Bovine Respiratory Syncytial Virus (BRSV) and Human Respiratory Syncytial Virus (HRSV) are closely related antigenically but have some differences. Humans and cattle are the main hosts of RSV, and the main transmission path of RSV is through nasal secretions, but it can also be transmitted through aerosols. There is little evidence to suggest that BRSV can be transmitted to humans and vice-versa for HRSV, as well as the fact that BRSV is a distinct virus from HRSV, and cattle are its natural host (Van der Poel et al., 1994; Makoschey & Berge, 2021).

However, the bacterial pathogens do carry a slightly increased, albeit minor, risk. The main risk comes from Tuberculosis (TB), which in turn is due to a small bacterium called Mycobacterium. There are many types of Mycobacteria but only two are transmissible between humans and cattle, *M. tuberculosis* and *M. bovis*. Both *M. tuberculosis* and *M. bovis* are treated with the same antibiotics, although not all *M. bovis* infections can lead to TB. In humans, symptoms of *M. bovis* are similar to those of *M. tuberculosis*. The primary route of transmission is through the exchange of respiratory secretions between the infected and uninfected patients, and persons at greatest risk are those who spend extended periods of time around cattle, those who have come into contact with cattle bodily fluids or tissue, and people who consume unpasteurized dairy products (Lombard et al., 2021).

While we will not be using any of these pathogens in our project, the proper course of action to take for preventing any transmission of BRD pathogens if we were to come into contact with cattle, would be proper hygiene such as hand-washing, refraining from touching eyes, mucous membranes, etc. with unwashed hands, and refraining from coming into contact with cattle bodily fluids as much as possible. Furthermore, our proof-of-concept will not use any animal pathogens, instead we will be utilizing BSL1 *E. coli* strains.

To address dual-use research of concern, the developed test kit would be sold specifically to the target user groups of veterinarians, farmers, and government professionals. However, the test kit could potentially be used by bad actors to identify and obtain these pathogens from infected

cattle for unlawful purposes. Proper safeguards and regulations on the use of the kit would be required.

The use of pre-approved tools, methods and procedures would be applied whenever possible, such as using the deep nasopharyngeal swabs which have already been validated as a collection method for BRD testing (Crosby et al., 2022). Additionally, the potential for false positives could cause undue alarm and could lead to the unwarranted culling of animals. Thorough testing of the test's specificity and sensitivity is necessary.

Finally, the proper disposal of used material is critical for our project's success. Nasal swabs used to collect the samples may be contaminated with BRD-causing bacteria. Clear protocols for safe disposal of biohazardous waste from testing is essential. We wish to make our kit as environmentally friendly as possible and hope to recycle some components. Therefore, stringent decontamination methods will have to be put in place to ensure no pathogens are spread between sites. Proper training of farm staff on safe handling and disposal is also essential.

Discussions

Bo-Find is advantageous in various ways in comparison to currently available solutions, largely owing to its ease of use. The system is fully contained in a user-friendly handheld device with color readout. This means the device can be used directly by the researchers or producers working with cattle, rather than relying on labs which consume more resources, incur higher costs, and can take multiple days for results to come back. Bo-Find takes less than an hour after initial testing onsite to give results. In addition to the convenience, a shorter detection time means the disease can be caught earlier before it proliferates, and action can be more effectively taken to treat affected cattle and stop the spread of disease. More effective detection can also lead to more targeted and efficient use of antibiotics given to cattle.

Bo-Find aligns with the United Nations' Sustainable Development Goals. The 12th Sustainable Development Goal calls for responsible consumption and production. Bo-Find addresses this by reducing the need for use of prophylactic antibiotics, which are overused on healthy cattle to prevent BRD. By detecting pathogens earlier, it allows for targeted treatment of only infected cattle rather than preventative treatment of the entire herd. Bo-Find also addresses the 13th Sustainable Development Goal, climate action. The testing kit will be primarily made from recycled and/or biodegradable materials, preventing high volumes of testing waste from entering landfills. If possible, we would also like to work with users to hold a recycling treatment initiative, which would allow us to collect used kits to be recycled for future use.

While superior in theory, the kit's design will need to be tested and improved for practical use. The device's current design allows for testing of only one cow at a time. This can make Bo-Find unfeasibly slow if large-scale detection is required, so we plan to find a way to improve the design to allow for faster testing of multiple animals at once. TwistDx currently holds a commercial license for use of RPA, which Bo-Find requires for its lack of temperature fluctuation needed during DNA amplification. To safeguard against license infringement, consultation with an intellectual property lawyer will be necessary before the product is released for commercial use. We will largely be testing the detection system using *E. coli*. While a safer and easier option than working with Bovine Respiratory Disease pathogens, the system may behave differently with the two bacteria. Due to this uncertainty, we will have to ensure before commercialization that the system effectively detects its intended pathogen.

To overcome these potential problems, we can consider developing a larger testing device that will be able to hold more rows on test, thus allowing for less waste while still testing the same amount of cattle. In feedlots cattle are usually divided into multiple pens. In these pens they are in close proximity to each other. Because of this, they develop very similar microbiomes within around 14 days from initial arrival at the feedlot. Consequently, only one to three cows would need to be tested per pen, depending on the size of the pen. This would allow for use of less tests, creating less product waste and less

time for farmers or researchers to test each cow.

Next Steps

While the RPA method for detecting BRDs has been established, our sample preparation method still needs validation. Our proof-of-concept will include creating a protocol that can be used in the field for easy DNA extraction (Figure 5). Typical cell lysis methods include mechanical disruption such as sonication, French press, or freeze-thaw methods which require specialized equipment. The design of the hardware and protocol capable of successful lysis of bacterial cells in the field requires testing to develop a lysis buffer stable at 100°C and a method to sufficiently extract the DNA from the cells.

The Bo-Find hand-held device will also require several rounds of testing and re-design. The first iteration of the device can be found here on TinkerCad: https://www.tinkercad.com/things/2aFWDiRsnzh-copy-of-bo-find. Working closely with members of the University of Lethbridge Agility Centre, we will print the device for testing. First, the device must be capable of being heated to 100°C, therefore the standard PLA used for 3D printing is not sufficient as it is not heat resistant. Testing using other polymers such as ABS or polypropylene will be needed. To build on this heat resistance, a biodegradable material such as Biomeht90 with a softening point of 110°C could provide a sustainable alternative to compounds listed above. Second, the device should be capable of sensing the colour change of the detection tubes. Our prototype will use a Raspberry Pi and RBG colour sensor. The goal is to be able to detect the colour shift from violet to blue in our samples upon DNA amplification. As an extension of colour detection, we hope to progress our technology onto the level of cellular devices. To do this, we will create an app which can instantaneously notify Bo-Find users of a colour change. This will improve the technology's ease of use and signify the end of our engineering cycle.

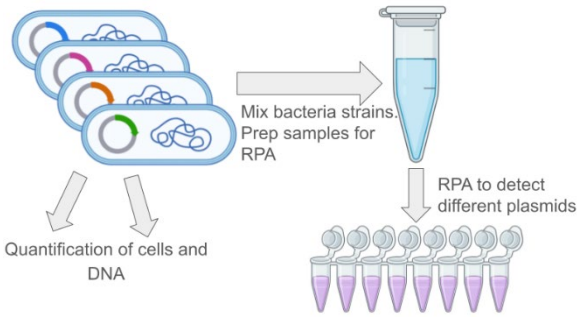


Figure 5. Overview for proof-of-concept testing using available E. coli strains. Bacteria cells containing plasmids with different fluorescent proteins (asPink and GFP, respectively) will be grown overnight and mixed at different concentrations, DNA extracted, and cells quantified. Extracted DNA will be used in downstream RPA reactions.

Author contributions

Video was produced by M.A, D.O., and N.S. Background section was prepared by J.C., J. B., J.W., G.A., A.B., R.B., N.B., E.B., M.W., and N.L. System, device and parts sections were prepared by L.K.W. Safety section was prepared by C.S., M.A., M.W., N.L., and I.B. The Discussions section was written by Z.W., N.B., E.B., Next steps were written by A.B.

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