# University of Wyoming Response to Joint Appropriations Committee Meeting December 14, 2021

# Submitted: January 7, 2022

**Follow-Up Items from JAC meeting on 12/14/2021:**

1. **Priority #1b Salary Increase Plan: Exit interviews- How many interviews were there of the 472 employees that left this year? and what are the primary reasons for employees leaving?**

**Overview of Reasons Behind UW's Record Outflow of Benefited Employees in 2021:**

1,850 benefited employees (1.7 per workday) left benefited positions at UW over the last 5 years. Of particular note, the annual outflow increased by nearly 70% since 2017:

| Year | Number of Benefited Employees Leaving UW |
|---|---|
| 2021 | 472 |
| 2020 | 371 |
| 2019 | 398 |
| 2018 | 326 |
| 2017 | 283 |

UW currently engages 2,861 benefited employees. Thus, UW's 2021 turnover (excluding internal transfers) is 16.5%.

Exit interviews conducted by UW Human Resources indicates that the majority - - nearly 60% - - of employees leaving UW do so primarily to accept non-UW employment. While UW does not ask departing employees about the role that salary plays in their decision to seek/accept alternative employment, these data would seem consistent with concerns that UW's salary levels are seen as being less competitive than other employers.

The next largest group - - 21% - - focused more on their experiences at UW (e.g., poor work environment, workload too heavy) in explaining their decision to leave UW.

The only other significantly large group - - 10% - - cited "lack of career advancement opportunities" as the primary factor in deciding to resign. This summer, UW Human Resources began creating 'career ladders' in various occupations to address this concern.

Two other factors mentioned by less than 5% of employees leaving UW were (a) moving out-of-state, and (b) COVID-19 impact on well-being and safety of UW employees.

**2. Priority #4- Office of Research and Economic Development- Could you please get a synopsis of the current activities and what economic development we are getting from the relationship with the Wyoming Business Council?**

# 2021 Wyoming Business Council Annual Report from the University of Wyoming: The Business Resource Network

## Introduction

A rapid evolution of synergies occurred in the State of Wyoming this past year. Constituents from across the state, including representatives from the University of Wyoming, the Wyoming Business Council, Wyoming's State Leadership, Business Leaders, Community Colleges and local communities worked collectively to rise up to the challenges of this extraordinary moment in Wyoming's economic history. A key innovation of this collaboration has been the emergence of the Wyoming Innovation Partnerships (WIP). Governor Gordon's leadership contributed to driving this concept forward. Governor Gordon, working with the University of Wyoming (UW) president, Ed Seidel, and the presidents of the state's Community Colleges are developing and acting on a vision that promises to capture a previously untapped potential of partnership. The WIP promises to create pathways for constructive engagement among Wyoming's Business Communities, Community Colleges, the University of Wyoming, and the Wyoming Business Council (WBC). Wyoming Innovation Partnerships will serve the needs of local communities while enabling a new class of responses to major economic forces and opportunities as they occur across the State, the Mountain West, and the Nation.

The Business Resource Network (BRN), supported in part by the WBC, federal sources, and UW, is comprised of economic development entities that UW envisions to be foundational elements of the WIP. The entities of the BRN strategically align with the WIP by offering a complementary range of services that are highly impactful, have a geographic distribution coinciding with hub WIP locations, a diverse range of support from state, federal, university, and local communities, as well as their growing ties with the regional Community Colleges. The BRN helps new businesses get started and helps established businesses continue to grow, every step of the way. Each of the three BRN units has distinguished itself as an important economic asset, including during the extended period of the pandemic. Regarding BRN units that were supported by the WBC in the second half of FY20 and continue to be supported in FY21, the following is of note:

- **IMPACT 307** has business incubators in Casper, Sheridan, Laramie and exploring opportunities in Cheyenne with the Laramie County Community College. This year IMPACT 307 successfully competed for federal funding that will enable extending its reach through the development of virtual business incubators at the seven community colleges and therefore at the locations of many of the WIP hub locations. This growing program captured the imagination of the U.S. Department of Commerce for its novelty and potential impact to rural America.

- **Manufacturing-Works** is Wyoming's arm of the national Manufacturers Extension Program of the Department of Commerce. It has performed at a high level, of such quality, that it has been recognized nationally by the former National Director of the MEP program. Its service areas already corresponds well with the envisioned reach of the WIP.
- **The Small Business Development Center Network** impacts have extended beyond its prescribed scope of work as it joined the WBC in serving Wyoming's businesses. Through extensive efforts during the pandemic Small Business Development Center Network connected vulnerable businesses to urgently needed federal resources. The areas it serves are also well aligned with the regions envisioned be served by the WIP.

The services provided by the BRN units range from emergent business incubation by IMPACT 307, small and new business support and resource identification from the Small Business Development Center Network, and manufacturing support through Manufacturing-Works. Such services will be relied upon heavily to support envisioned state-wide WIP activities.

A key element of the BRN's activities, and also those activities that the WIP will support, is to support the goals of the WBC's strategic plan. This report is divided into four sections, one for each BRN unit and provides a representation of how each unit serves the various initiatives encompassed within the WBC's strategic plan (listed in *blue italics*). In each section we have provided metrics for this reporting year that speak to the impacts to the WBC plan's goals. Some of these metrics, while not requested specifically by the WBC, are required to be reported to federal agencies that support the work of the unit and are grounded in definitions provided by these agencies. Also included are a sampling of testimonials from clients that IMPACT 307, Manufacturing-Works, and the Small Business Development Center Network serve. Of note is that UW's Technology Transfer and Research Products Center is now exclusively supported by UW. This unit of the BRN received some support from the WBC in months prior to the contract modification in the middle of FY20, therefore a summary of its relevance to the WBC strategic plan is also provided.

# The WBC-State Economic Development Strategy Alignment – IMPACT 307

***The WBC-State Economic Development Strategy Purpose*** *- Creating new opportunities for current and future generations of Wyomingites by ADDING VALUE to Wyoming's core industries and LEVERAGING them to ACTIVATE new economic sectors*

**IMPACT 307 Purpose** - IMPACT 307 focuses on experiential teaching of founders, building entrepreneurial support ecosystems across Wyoming, improving entrepreneurial knowledge and business wisdom, coupled to the University of Wyoming's Land-Grant university charter of serving the state.

**IMPACT 307 Mission -** Through direct intervention, IMPACT 307 assists individuals and early-stage, growth-oriented Wyoming companies, which have the potential of becoming primary employers in the state. IMPACT 307 assists in defining the new business or product models from the concept stage through mature growth by teaching, mentoring, networking, and advising.

- IMPACT 307 figuratively becomes a lead director on all client company boards, guiding, assisting, and adjusting company business models for long-term success.
- IMPACT 307 is not *Process*-oriented, but rather *Outcomes*-oriented through founders and people.

***The WBC-State Economic Development Strategy Vision*** *– Realize diverse, broad and lasting growth so that Wyoming can prosper no matter the economic climate or status of individual sectors*

**IMPACT 307 Vision** – Establishing a Wyoming growth-oriented business ecosystem where entrepreneur ventures diversify the economy of the state while concurrently maximizing their own sustainable growth.

| *WBC Initiative* | IMPACT 307 Supporting Activities and Execution |
|---|---|
| ***Add Value to Core Industries*** *natural resources, tourism and outdoor recreation, agriculture* | IMPACT 307 is industry-agnostic on a statewide scale; all of the listed industry sectors are served; all jobs are good jobs.<br>• Using the community college districts as a regional map, each region has a unique business demographic mix. Laramie has a strong technology sector due to the University of Wyoming. Sheridan has a strong light manufacturing sector. Casper has a very strong historical oil and gas sector as well as a growing medical dominance in the state. Each city and region will |

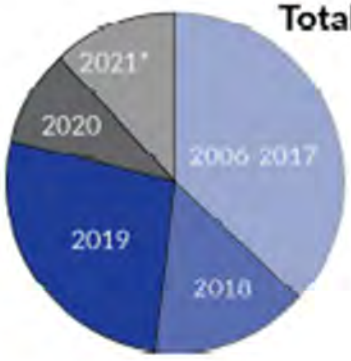
| *WBC Initiative* | IMPACT 307 Supporting Activities and Execution |
|---|---|
| ***Add Value to Core Industries*** *natural resources, tourism and outdoor recreation, agriculture (cont.)* | have its own flavor of businesses, one which will likely evolve over time.<br><br>**EXECUTION Examples:**<br>Even in a year of COVID, and challenges getting engagement from entrepreneurs in both the University of Wyoming and across the State, the IMPACT team assisted the startup of 25 new businesses, most with a technology or innovation focus. Less than a fifth were related to core industries but instead were diversification efforts establishing new solutions to identified problems in new economic sectors (WBC Initiative number 2).<br>• Included in the core industry startups are two that are related to cattle, stock and rangeland management as well as a lumber operation.<br><br>• IMPACT 307 wrote and was awarded a large Federal EDA grant that expands IMPACT 307's ability to cover underserved areas of the state. This and the addition of now-statewide "virtual incubator" coverage, is a scope commitment to double the number of startup challenges across the state from 5 to 10-12. The startup challenges will target historically underserved communities like Gillette, Cody/Powell, Torrington, Fremont County, and Green River/Rock Springs. |
| ***Activate New Economic Sectors*** *healthcare; financial, scientific, and professional services; digital and technology; arts and culture; advanced manufacturing* | IMPACT 307 supports a broad range of sectors, including emerging markets, technologies, and innovation sectors due to its regional, entrepreneurial, cultural, and historical strengths.<br>• IMPACT 307 does not make judgements or give pre-consideration to acceptable industries or segment models; all potentially have merit. IMPACT 307's responses are guided generally by execution and principals, not the target industry.<br>• IMPACT 307 endeavors to assist all growth-oriented entrepreneurial businesses regardless of which industry(s) or market(s) they operate.<br>• IMPACT 307 retains a diverse mix of advisors with experience across multiple industries, segments, and demographics.<br>• Working within the IMPACT 307 network, Business Resource Network (BRN), and the University of Wyoming overall, there is not a business, development, or technology segment that has an advisor gap. |

| *WBC Initiative* | IMPACT 307 Supporting Activities and Execution |
|---|---|
| ***Activate New Economic Sectors*** *healthcare; financial, scientific, and professional services; digital and technology; arts and culture; advanced manufacturing (cont.)* | **EXECUTION Examples:**<br>IMPACT 307 continues to be the principal driver of the Wind River Startup Challenge (WRSC). Multiple local stakeholders are assisting IMPACT 307 in raising award funding and now the WRSC has sustaining award funding for at least the next two years.<br>Technology startups, as cited in the WBC Initiative statement in this category, mainly flow out of University of Wyoming spinouts. That's not exclusive, as Casper has also assisted new ventures in that region associated with healthcare and digital technology.<br>• One of IMPACT 307's life science startups recently had proof experiments launched in zero-G on the last SpaceX mission that went up in June 2021. This startup is targeting stabilizing vaccines outside of the cold chain (e.g., Pfizer/Moderna mRNA vaccines for COVID) by using encapsulation technology adapted from natural products found in Wyoming. Success would lead to massive global health benefits. |
| ***Local Economic Development***<br>▪ *Foster a culture of regional connectedness*<br>▪ *Empower communities to sustainably develop their economies*<br>▪ *Work with all partners to develop effective approaches to Economic Development* | IMPACT 307 and the University of Wyoming overall will work closely with all of the State of Wyoming's Community Colleges with the common goal of promoting entrepreneurship, innovation, and economic resilience.<br><br>IMPACT 307 and its program will be a significant part of Governor Gordon's Wyoming Innovation Partnership (WIP) to diversify Wyoming's economy and strengthen its workforce.<br><br>IMPACT 307 has developed a strategic relationship with the Wyoming Economic Development Association (WEDA) and will work with all community EDOs in the state.<br><br>IMPACT 307 works with local communities, in, around and outside of where the physical centers are located. IMPACT 307 provides guidance, counseling, and direction in empowering community members to catalyze entrepreneurial efforts through the program's startup challenges. Through these efforts there is a focus on developing swift, substantial, and cost-effective results.<br><br>IMPACT 307 will work to grow the innovation hub concept within WIP as described by University of Wyoming President Seidel and Governor Gordon. |

| *WBC Initiative* | IMPACT 307 Supporting Activities and Execution |
|---|---|
| ***Local Economic Development***<br>▪ *Foster a culture of regional connectedness*<br>▪ *Empower communities to sustainably develop their economies*<br>▪ *Work with all partners to develop effective approaches to Economic Development*<br>*(cont.)* | **EXECUTION Examples:**<br>IMPACT 307 is already developing specific working agreements with four of the seven Community Colleges in Wyoming; establishing a relationship of trust with the remaining three before moving further ahead.<br><br>IMPACT 307 is working closely with the community of Gillette to establish a stronger presence within the Gillette FUEL incubator and in establishing a formal program for entrepreneurial mentoring and guidance at that location. |
| ***Partnerships***<br>▪ *Coordinate inter-agency plans and programming*<br>▪ *Align advisory boards*<br>▪ *Increase communication across partnerships*<br>▪ *Increase alignment and clarity of roles and responsibilities.* | IMPACT 307 works with all local EDO's to leverage and facilitate the program's offerings; they are a crucial partner to success.<br><br>IMPACT 307 works with all seven regional community colleges in the State of Wyoming.<br><br>IMPACT 307 seeks partnership with the Wyoming Business Council to help meet relevant parts of the WBC's mission.<br><br>IMPACT 307 works in close coordination with the other BRN units of the University of Wyoming to efficiently provide the required expertise and resources needed to serve emerging Wyoming businesses.<br><br>**EXECUTION Examples:**<br>IMPACT 307's roles and responsibilities are directly related to its overall Purpose and Mission (see narrative at the beginning of this section) and has remained unchanged for at least a decade.<br><br>IMPACT 307 does not have a formal advisory board, however it does have multiple stakeholders. With respect to the latter, consistent messaging, transparency, consistent execution, and continuous improvement are hallmarks of the overall philosophy and program.<br><br>IMPACT 307 continues to partner and have close relationships with the Wyoming Economic Development Association and with the Wind River Development Fund. |

| *WBC Initiative* | IMPACT 307 Supporting Activities and Execution |
|---|---|
| ***Expertise***<br>▪ *Leverage industrial expertise to add value (to) core industries and activate new ones.*<br>▪ *Increase functional expertise in key area of economic development, innovation, supply chain, startups, market development, workforce, finance, etc.* | IMPACT 307's staff experience covers a wide range of fields across various entrepreneurial needs as shown as a need in startup competitors and businesses.<br>• These fields include the areas of: finance, accounting, graphic arts, marketing, project management, pharmaceutical industry, business processes, manufacturing, engineering, capital equipment, software,<br>High-tech, IP portfolio management, new product development and introduction, product lifecycle management, operations, etc.<br><br>The executive experience of the IMPACT 307 staff ranges from CFO to CTO to General Manager and to a VP of Engineering.<br><br>**EXECUTION Examples:**<br>IMPACT 307 added three new experienced Assistant Directors, one new Business Coordinator, and promoted an existing staff member to Marketing Coordinator in 2021.<br>• Experience diversity among IMPACT 307 staff remains high, those newly hired advisors include a chemist, an economic development professional, and serial entrepreneur who is also an attorney. |

## IMPACT 307 PERFORMANCE DATA




**Total Companies Launched: 219**
2006-2017: 81
2018: 33
2019: 59
2020: 20
2021: *26
*as of 9/1/2021

# The WBC-State Economic Development Strategy Alignment – Manufacturing-Works

***The WBC-State Economic Development Strategy Purpose*** *- Creating new opportunities for current and future generations of Wyomingites by ADDING VALUE to Wyoming's core industries and LEVERAGING them to ACTIVATE new economic sectors*

**Manufacturing-Works Purpose** - Manufacturing-Works helps Wyoming manufacturers, producers and entrepreneurs succeed. The core focus is to help manufacturers identify their weaknesses and deliver solutions to increase efficiency and profitability while reducing the bottlenecks that get in the way of routine business operations.

**Manufacturing-Works Mission -** Manufacturing-Works is Wyoming's premier non-profit, technical consultancy. Assisting Wyoming Manufacturers and Producers of every size and type in ALL aspects of improving top-line potential and bottom-line growth. The staff at Manufacturing-Works are highly skilled and experienced professionals with direct access to a network of local and nationwide expertise. As a consultancy Manufacturing-Works does much more than advise clients, the staff facilitates tailored, viable solutions for clients; and when necessary, oversees implementation. Given the expertise and the power of Manufacturing-Works network, there is not a technical problem or business challenge for which the staff at Manufacturing-Works cannot facilitate a solution.

***The WBC-State Economic Development Strategy*** *– Realize diverse, broad and lasting growth so that Wyoming can prosper no matter the economic climate or status of individual sectors*

**Manufacturing-Works Vision** - Empowering Wyoming manufacturers, by assisting them in adopting productivity enhancing, innovative manufacturing technologies, navigating advanced technology solutions, and developing a skilled and diverse workforce. Manufacturing-Works acts to champion manufacturing by promoting the importance of a strong Manufacturing base in Wyoming, creating awareness of innovations in manufacturing and enabling workforce development partnerships. Manufacturing-Works leverages partnerships at the national, regional, state, and local levels. This increases market penetration, identifies mission-complementary advocates to help expand the brand recognition of the Wyoming MEP (The Holling's Manufacturing Extension Partnership) and builds an expanded service delivery model to support manufacturing technology advances.

| *WBC Initiative* | Manufacturing-Works Supporting Activities and Execution |
|---|---|
| ***Add Value to Core Industries*** *natural resources, tourism and outdoor recreation, agriculture* | Manufacturing-Works as defined by our contract with NIST MEP focuses almost solely on facilitating performance enhancing services to Wyoming's small and medium manufacturers.<br><br>A summary report to Congress states that "The Hollings Manufacturing Extension Partnership (MEP) program is a national network of centers established by the Omnibus Trade and Competitiveness Act (P.L. 100-418).<br>• MEP centers provide custom services to small and medium-sized manufacturers (SMMs) to improve production processes, upgrade technological capabilities, and facilitate product innovation. Operating under the auspices of the National Institute of Standards and Technology (NIST), the MEP system includes centers in all 50 states and Puerto Rico."<br><br>**EXECUTION Examples:**<br>Manufacturing-Works worked closely with a Wyoming leading manufacturer of lightweight, sub-MOA, long-range custom hunting rifles to assist in market expansion. The project resulted in the creation of 2 primary jobs, 50% increase in contract backlog, ≈ $300,000 in increased annual revenue and significant penetration into new markets.<br><br>Early in the COVID-19 Pandemic, a shortage in PPE was quickly realized. Manufacturing-Works worked closely with Wyoming Makers Spaces to facilitate a $48,000 Community Development Block Grant and a $17,000 Community Foundation Grant to ramp up production of face masks and other PPE. |
| ***Activate New Economic Sectors*** *healthcare; financial, scientific, and professional services; digital and technology; arts and culture; advanced manufacturing* | In the wake of COVID-19 and the vulnerabilities exposed in the global supply chain, Manufacturing-Works heavily focused on connecting Wyoming manufacturers to the regional, national, and global markets.<br><br>Advanced manufacturing technology services, or Industry 4.0 solutions that provided by Manufacturing-Works include: Additive Manufacturing/3D Printing, Augmented and Virtual Reality, Big Data, Cloud Computing, Cybersecurity, Internet of Things, Robotics/Flexible Automation, Simulation, and System Integration. |

| *WBC Initiative* | Manufacturing-Works Supporting Activities and Execution |
|---|---|
| ***Activate New Economic Sectors*** *healthcare; financial, scientific, and professional services; digital and technology; arts and culture; advanced manufacturing (cont.)* | **EXECUTION Examples:**<br>Manufacturing-Works is working closely with Wyoming manufacturers to leverage new technologies and enhance their capabilities. The Manufacturing-Works team has developed an education and demonstration platform that assists in evaluating the ROI on switching manual processes to automated processes. Close work is being done with Makers Spaces throughout the state to demonstrate the effectiveness of automation to manufacturers throughout the state. |
| ***Local Economic Development***<br>▪ *Foster a culture of regional connectedness*<br>▪ *Empower communities to sustainably develop their economies*<br>▪ *Work with all partners to develop effective approaches to Economic Development* | All Manufacturing-Works territory project managers actively engage with relevant economic development partners, which include organizations such as WBC regionals, local Chambers of Commerce and WEDA to name a few.<br><br>Manufacturing-Works feels that open, honest, and collaborative communication is at the core of reducing duplication of efforts and successfully fulfilling our respective missions.<br><br>**EXECUTION Examples:**<br>The Northeast Manufacturing Works Project Manager worked in closely with many partners including: the WBC, Wyoming Workforce Services, City of Sheridan, Sheridan Chamber of Commerce, Impact 307, WY DHS, the Wyoming SBDC Network, the University of Wyoming and the local non-profit Phorge Makerspace, to apply and successfully receive grant money to produce PPE for the State of Wyoming. This effort was put forth as current mutual need existed. Now there are established working relationships that are mutually beneficial going forward.<br><br>Manufacturing-Works communication with the Economic Development Organization (EDO) stakeholders happens on a daily basis, across the whole State of Wyoming. Team members visit local Chambers of Commerce and other community economic developers regularly. Depending on county and city, Their meeting may cover topics such as: new business moving in to town, committee meetings, collaboration on new and existing grants and funding methods, client referrals, new deliverables and resources, and sometimes about future hurdles where a proactive approach is needed. Manufacturing-Works philosophy is to work across boundaries, with all stakeholders, |

| *WBC Initiative* | Manufacturing-Works Supporting Activities and Execution |
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| ***Local Economic Development***<br>▪ *Foster a culture of regional connectedness*<br>▪ *Empower communities to sustainably develop their economies*<br>▪ *Work with all partners to develop effective approaches to Economic Development (cont.)* | rather than solo so as to foster mutually beneficial relationships that benefit SMMs throughout the state. |
| ***Partnerships***<br>▪ *Coordinate inter-agency plans and programming*<br>▪ *Align advisory boards*<br>▪ *Increase communication across partnerships*<br>▪ *Increase alignment and clarity of roles and responsibilities.* | Manufacturing-Works partners with relevant Economic Development Officers to leverage and facilitate services. By doing so, Manufacturing-Works is better able to educate manufacturers of relevant changes in the manufacturing sector that might trigger a positive or negative trend in industry. Manufacturing-Works collaborates with all of Wyoming's Community Colleges. Together the combination aspires to educate the appropriate stakeholders about future workforce development and manufacturing jobs as a viable career path.<br><br>Manufacturing-Works partners with the Wyoming Business Council to help meet relevant parts of the Councils' mission.<br><br>Manufacturing-Works collaborates with other University of Wyoming BRN units to efficiently provide required expertise and resources to serve Wyoming manufacturers.<br><br>**EXECUTION Examples:**<br>At the beginning of the COVID-19 shutdown, Manufacturing-Works worked swiftly to develop, plan, and execute business health and recovery webinars for Wyoming Manufacturers. These focused on topics such as: how to work virtually, what funds were available related to recovery, how and where to apply for these funds.<br><br>Manufacturing-Works also holds an active role with Workforce Advisory Groups at the Community Colleges throughout the State of Wyoming.<br>• Manufacturing-Works participated in the development of plans for Laramie County Community College's proposed Advanced Manufacturing and Materials Center. |

| *WBC Initiative* | Manufacturing-Works Supporting Activities and Execution |
|---|---|
| ***Investments and Services***<br>▪ *Revise programs to create investments with measured returns*<br>▪ *Develop services that clearly meet the needs of business, industry, and partners*<br>▪ *Regularly review performance and adjust the portfolio and budget accordingly.* | Manufacturing-Works has a relevant portfolio of service offerings. The offered services align with the needs of Wyoming's manufacturers that want to expand, develop and become competitive in the manufacturing markets outside of traditional energy and mineral extraction.<br><br>**EXECUTION Examples:**<br>As a result of COVID-19 and the resulting supply chain issues, Manufacturing-Works worked closely with Wyoming distillers throughout the state to manufacture and distribute much needed hand sanitizer to local governments, businesses, and healthcare entities. |
| ***Expertise***<br>▪ *Leverage industrial expertise to add value (to) core industries and activate new ones.*<br>▪ *Increase functional expertise in key area of economic development, innovation, supply chain, startups, market development, workforce, finance, etc.* | Manufacturers just like federal, state and local agencies need access to critical products to ensure our nation's health, safety and economic security. Many of these products are in short supply.<br>• Small and medium-sized manufacturers (SMMs) are especially challenged with sustaining their operations in light of current conditions. Currently, there is a nationwide shortage of domestic manufacturing supplies necessary to meet the needs of the SMMs. It is anticipated that the shift to a regional-United States centric supply chain is a problem that will persist for the foreseeable future. Manufacturing-Works in conjunction with the MEP National Network are facilitating in the efforts to locate the needed supplies.<br><br>Manufacturing-Works connects Wyoming manufacturers with relevant production capabilities with other manufactures who have capacity which enables both to fulfill current market and societal needs. Manufacturing-Works then assists those manufacturers in delivering needed products.<br><br>**EXECUTION Examples:**<br>Manufacturing-Works regularly participates in NEXGEN partnerships throughout the State of Wyoming related specifically to manufacturing. The NEXGEN groups include industry partners, EDOs, the WBC, and other stakeholders with a vested interest in manufacturing. Manufacturing-Works brings available information about nationwide industry trends, challenges, and successes to the table. With this information, the manufacturing community in Wyoming is able to proactively |

| *WBC Initiative* | Manufacturing-Works Supporting Activities and Execution |
|---|---|
| ***Expertise***<br>▪ *Leverage industrial expertise to add value (to) core industries and activate new ones.*<br>▪ *Increase functional expertise in key area of economic development, innovation, supply chain, startups, market development, workforce, finance, etc. (cont.)* | plan and address current and upcoming issues facing the manufacturing industry. |

## MANUFACTURING-WORKS PERFORMANCE DATA

(Metrics reported to The Holling's Manufacturing Extension Partnership (MEP) based on clients surveyed in FY20)

| Total Increased/Retained Sales | Total Increased/Retained Jobs | New Client Investments | Cost Savings |
|---|---|---|---|
| $3.4 Million | 65 | $2.1 Million | $292K |




# WBC/ State Economic Development Strategic Alignment – Wyoming SBDC Network

(Small Business Development Center (SBDC), Procurement Technical Assistance Center (PTAC), Wyoming SBIR/STTR Initiative (WSSI), Market Research Center (MRC))

***The WBC-State Economic Development Strategy Purpose*** *– Creating new opportunities for current and future generations of Wyomingites by ADDING VALUE to Wyoming's core industries and LEVERAGING them to ACTIVATE new economic sectors*

**Wyoming SBDC Network Purpose -** The SBDC is a source of technical and managerial assistance for small businesses in Wyoming and across the United States. The mission of a Small Business Development Center (SBDC) is to promote growth, innovation, productivity, and revenue for small businesses through improvements to their business administration. The Wyoming SBDC Network serves businesses, entrepreneurs and startups, industries of all types, and communities so businesses can thrive, survive, and contribute to the diversification of Wyoming's economy through a variety of services and approaches:

- Provides no-cost, confidential, individualized support and connections to resources and tools that help businesses pivot, grow, thrive, and become resilient in changing markets and economies.
- Cultivates and provides the culture, networking opportunities, and resources that help entrepreneurs start and grow businesses.
- Develops and provides connections to resources and enablers like workforce, innovation, research, and training that help target industries grow and expand.
- Delivers accessible services, tools, opportunities, and amenities that enable growth of local economies and participation in global economies.

**Wyoming SBDC Network Mission -** The Wyoming SBDC Network helps businesses succeed.

***The WBC-State Economic Development Strategy Vision*** *– Realize diverse, broad and lasting growth so that Wyoming can prosper no matter the economic climate or status of individual sectors*

**Wyoming SBDC Network Vision -** Wyoming's leading provider of business expertise.

| *WBC Initiative* | Wyoming SBDC Supporting Activities and Execution |
|---|---|
| ***Add Value to Core Industries*** *natural resources, tourism and outdoor recreation, agriculture* | The Wyoming SBDC Network works with all industries, at all stages of the life of a business, including those targeted by the WBC/State Economic Development Strategy initiative. |

| *WBC Initiative* | **Wyoming SBDC Supporting Activities and Execution** |
|---|---|
| ***Add Value to Core Industries*** *natural resources, tourism and outdoor recreation, agriculture (cont.)* | Regional directors/business advisors are dispersed in regions around the state to meet clients where they are geographically. In addition, each regional director and advisor possesses a unique area of expertise (marketing, finance, international trade, government contracting, accounting software, human resources management, cybersecurity, etc.) that can be accessed by clients throughout the state. Advisors often employ a team approach to provide clients the highest degree of technical assistance possible to meet their unique business needs.<br><br>**EXECUTION Examples:**<br>During FY21, the Wyoming SBDC Network assisted businesses in these strategically-identified sectors (Note: Not all of the strategic categories are mirrored in our database categories; we have selected the categories that are most comparable):<br><br>**Accommodation and Food Services:** Assisted 133 total businesses that employed 565 full-time and 569 part-time employees with annual reported sales of nearly $61 million*. Of those businesses, 11 were startups employing 32 full-time and 29 part-time employees with reported sales of just over $1million**.<br><br>**Agriculture, Fishing, and Hunting:** Assisted 62 total businesses employing 109 full-time and 70 part-time employees with annual reported sales of over $4.5 million. Of those businesses, 9 were startups employing 40 full-time and 23 part-time employees with reported sales of $125,000.<br><br>**Mining:** Assisted 12 total businesses employing 109 full-time and 6 part-time employees with annual reported sales of over $26 million. Of those businesses, 1 was a startup employing 2 full-time employees with no reported sales.<br><br>*For only the sectors noted above, MRC provided 142 hours of market research for 9 companies, PTAC provided 75 hours of client assistance for 21 companies, and WSSI provided 8 hours of assistance for 1 company.<br><br>* *Only about half of clients report annual sales.*<br>** *Even fewer startups report annual sales as they have not been in business long enough to have a sales history.* |

| *WBC Initiative* | Wyoming SBDC Supporting Activities and Execution |
|---|---|
| ***Activate New Economic Sectors*** *healthcare; financial, scientific, and professional services; digital and technology; arts and culture; advanced manufacturing* | The Wyoming SBDC Network is technology-accredited by our national association, allowing us to provide specialized assistance to clients with intellectual property, cybersecurity, and technological innovation needs. Advisors work closely with our WSSI program manager to assist clients in pursuing federal SBIR and STTR funding opportunities to undertake innovation-focused research, develop technology products, and make appropriate referrals to additional resources.<br><br>The Wyoming SBDC Network's Market Research Center provides crucial competitive intelligence data to clients wishing to enter emerging markets in all industries noted in the WBC/Statewide initiatives. Market research data allows clients to make crucial business startup or expansion decisions based on key economic factors, market opportunities, and industry-specific data.<br><br>**EXECUTION Examples:**<br>During the reporting period, the Wyoming SBDC Network assisted businesses in these strategically-identified sectors:<br><br>**Health Care and Social Assistance:** Assisted 102 total businesses that employed 450 full-time and 143 part-time employees with annual reported sales of just over $38 million. Of those businesses, 13 were startups employing 13 full-time and 4 part-time employees with reported sales of $110,000.<br><br>**Finance and Insurance:** Assisted 17 total businesses that employed 68 full-time and 11 part-time employees with annual reported sales of nearly $2.4 million. Of those businesses, 3 were startups employing 3 full-time employees with no reported sales.<br><br>**Professional, Scientific, and Technical Services (including digital and technology-oriented businesses):** Assisted 159 total businesses that employed 680 full-time and 134 part-time employees with annual reported sales of nearly $30 million. Of those businesses, 11 were startups employing 8 full-time and 4 part-time employees with reported sales of $280,000.<br>**Arts, Entertainment, and Recreation:** Assisted 99 total businesses that employed 155 full-time and 138 part-time employees with annual reported sales of nearly $9.3 million. Of those businesses, 11 were startups employing 11 full-time and 9 part-time employees with reported sales of $12,700. |

| *WBC Initiative* | Wyoming SBDC Supporting Activities and Execution |
|---|---|
| ***Activate New Economic Sectors*** *healthcare; financial, scientific, and professional services; digital and technology; arts and culture; advanced manufacturing (cont.)* | **Manufacturing:** Assisted 157 total businesses that employed 1,210 full-time and 181 part-time employees with annual reported sales of nearly $166.5 million. Of those businesses, 11 were startups employing 7 full-time and 6 part-time employees with reported sales of $420,000.<br><br>**For only the sectors noted above, MRC provided 962 hours of market research for 68 companies, PTAC provided 523 hours of client assistance for 70 companies, and WSSI provided 250 hours of assistance for 21 companies. WSSI specializes in providing advising and assistance to technology-oriented companies and those seeking to commercialize intellectual property and is a key player in the state's economic efforts to develop technology-oriented businesses.* |
| ***Local Economic Development***<br>▪ *Foster a culture of regional connectedness*<br>▪ *Empower communities to sustainably develop their economies*<br>▪ *Work with all partners to develop effective approaches to Economic Development* | The Wyoming SBDC Network fosters a culture of regional connectedness through ongoing collaboration with WBC field staff, economic development entities, lending institutions, and community organizations at a regional level. Each regional director is skilled at networking and maintains strong relationships with economic development and community-based partners.<br><br>The Wyoming SBDC Network empowers communities to develop their own economies by working with all types of community-based businesses who request assistance and by proactively offering services to existing businesses. The Wyoming SBDC Network works with small businesses of all sizes and types, including those providing essential amenities and services, such as health and child care, recognizing that a community offering such resources makes it more attractive to larger businesses wishing to locate to the area. The Wyoming SBDC Network believes that every job is a good job and even the most modest small business provides a sense of independence and fulfillment to its owners and a strength to its community.<br><br>The Wyoming SBDC Network does not provide its services in a vacuum and often serves as a hub to direct clients to other state, regional, and local economic development resources. Our strong networking efforts result in a broad-based, partnered approach to regional economic development. |

| *WBC Initiative* | **Wyoming SBDC Supporting Activities and Execution** |
|---|---|
| ***Local Economic Development***<br>▪ *Foster a culture of regional connectedness*<br>▪ *Empower communities to sustainably develop their economies*<br>*Work with all partners to develop effective approaches to Economic Development (cont.)* | **EXECUTION Examples:**<br>During the program period, Wyoming SBDC Network staff took part in the following sampling of activities to participate in and leverage local and regional economic development efforts:<br><br>**Foster a culture of regional connectedness:**<br>Regional directors throughout the state attend monthly, in-person networking events or virtual meetings with area economic and community development resources in the counties they serve, including WBC field staff, city leaders, chambers, and other ED agencies.<br>• A regional director meets virtually with the WBC export development team to discuss economic diversification activities and efforts and supports the WBC STEP program.<br>• A regional director and PTAC program manager presented a resource section to the Colorado/Wyoming Colossal Colorado Virtual Industry Event hosted by the Society of American and Military Engineers.<br>• The SBDC participates in the annual Wyoming Bankers Credit Conference either with a booth and networking or with a formal presentation.<br>**Empower communities to sustainably develop their economies:**<br>A regional director assists in overseeing the Rainbow Teton Entrepreneurship Center, the small business incubator in Rawlins.<br><br>A regional director works with Central Wyoming College and Impact 307 to provide classes, one-on-one training, and preparation assistance to participants of the Wind River Startup Challenge. Another regional director served as a judge for the competition.<br><br>A regional director regularly attends a local high school's Career and Technical Education meetings in the spring and fall of each year to share information from the business community in terms of desired skills and qualities needed for the modern workforce. |

| *WBC Initiative* | **Wyoming SBDC Supporting Activities and Execution** |
|---|---|
| ***Local Economic Development***<br>▪ *Foster a culture of regional connectedness*<br>▪ *Empower communities to sustainably develop their economies*<br>*Work with all partners to develop effective approaches to Economic Development (cont.)* | **Work with all partners to develop effective approaches to economic development:**<br>All SBDC advisors worked closely during the height of the pandemic with both the WBC regional directors and SBA personnel to assist clients encountering issues obtaining pandemic funding or problems with having those funds forgiven. Relief funding efforts continue as such programs progress. Additionally, we continue to collaborate with our partners to serve client needs as new funding opportunities become available.<br>• The SBDC regularly participates in SBA recognition programs and nominates clients for award consideration.<br>• A regional director collaborates with the EXIM Bank regional director and the SBA international trade specialist in the promotion of financial solutions to Wyoming exporters and other international trade issues.<br>• Market Research Center Staff worked on exploring database and resource collaborations with UW Coe Libraries, Wyoming State Libraries, and the Laramie County Library system.<br>• An SBDC advisor is featured on the WBC website as the state's licensing and permitting expert. The advisor fields dozens of inquiries annually that require extensive research on a variety of regulation topics.<br>*These are just a few examples of specific and ongoing activities to support this strategic initiative. Many more instances can be found in our latest SBA Semi-Annual Report, specifically categories 1600: Advocacy, 1700: Resource Development, and 1800: Collaboration and Leveraging (pages 19-22). (WBC staff was copied on the e-mail submission of the report on July 30, 2021.)* |
| ***Partnerships***<br>▪ *Coordinate inter-agency plans and programming*<br>▪ *Align advisory boards*<br>▪ *Increase communication across partnerships*<br>▪ *Increase alignment and clarity of roles and responsibilities.* | In addition to the partnerships mentioned above, the Wyoming SBDC Network regularly participates in WEDA events and webinars to ensure our efforts are in pursuit of statewide economic development goals. We also maintain strong relationships with WBC field staff and attend WBC board meetings to ensure our strategic plans remain aligned.<br>Our advisory board members are located throughout the state and are aware of statewide economic development efforts and provide |

| *WBC Initiative* | **Wyoming SBDC Supporting Activities and Execution** |
|---|---|
| ***Partnerships***<br>▪ *Coordinate inter-agency plans and programming*<br>▪ *Align advisory boards*<br>▪ *Increase communication across partnerships*<br>▪ *Increase alignment and clarity of roles and responsibilities. (cont.)* | advocacy, guidance, and advice to develop and deploy coordinated programming.<br><br>A member of the WBC staff and representatives of the SBA district office attend our weekly network call with all SBDC staff. Our training opportunities and program-specific news are shared regularly with all economic development and legislative entities throughout the state.<br><br>Through ongoing networking, marketing, and communication-sharing opportunities, the Wyoming SBDC Network makes stakeholders and potential clients aware of the array of services and expertise available. When appropriate, clients are referred to other partners who possess specific expertise, not only to Business Resource Network partners, but others such as the Secretary of State, Main Street associations, local lenders, Chambers, Workforce Services, regional economic development entities, etc.<br><br>**EXECUTION Examples:**<br>In the spirit of keeping the length of this report manageable, the descriptions listed above contain examples of our ongoing activities to maintain vital economic development partnerships.<br><br>**For further detail, refer to the SBA Semi-Annual Report, specifically categories 1600: Advocacy, 1700: Resource Development, and 1800: Collaboration and Leveraging (pages 19-22).* |
| ***Investments and Services***<br>▪ *Revise programs to create investments with measured returns*<br>▪ *Develop services that clearly meet the needs of business, industry, and partners*<br>▪ *Regularly review performance and adjust the portfolio and budget accordingly.* | The Wyoming SBDC Network staff undergoes an annual, rigorous strategic planning process to evaluate programming and service efforts, communication and collaboration with partners, and to investigate new programming and operational opportunities to continually improve our offerings.<br>We continually survey clients to evaluate our efforts and make changes to programming and services as needed. We also informally and formally survey stakeholders to measure performance and consider improvements. More important than formal survey results to gauge effectiveness are the ongoing individual relationships we maintain with clients and the strong networking we cultivate with our partners and stakeholders. |

| *WBC Initiative* | **Wyoming SBDC Supporting Activities and Execution** |
|---|---|
| ***Investments and Services***<br>▪ *Revise programs to create investments with measured returns*<br>▪ *Develop services that clearly meet the needs of business, industry, and partners*<br>▪ *Regularly review performance and adjust the portfolio and budget accordingly. (cont.)* | As mentioned in the previous point, performance review is ongoing with clients and stakeholders. Internally, we review performance milestones as an organization monthly and adjust strategic and operational approaches as needed to meet and exceed our goals. Additionally, the SBDC has annual site reviews conducted by the state's Small Business Administration (SBA) project officer, bi-annual programmatic and financial reviews performed by the U.S. SBA program manager and Office of Entrepreneurial Development Financial Examiner, respectively.<br><br>**EXECUTION Examples:**<br>**Revise programs to create investments with measured returns:** All members of the Wyoming SBDC Network attended virtual strategic planning sessions June 1-4, 2021. Staff formed work groups to review progress on the previous year's strategic goals and tasks and revised and amended as necessary to meet current and anticipated program needs. During this year's sessions, advising staff also participated in a training webinar presented by the Commit Foundation to receive information and strategies for providing better service to veterans.<br><br>All SBDC staff participate in a network-wide virtual meeting every Tuesday morning to receive updates on programs and review operational topics to ensure consistency in program delivery and to make sure all staff have the most current information to do their jobs effectively. All staff also participate in weekly small group "pod calls" led by the program administers to discuss specific programmatic or operational topics and to inform other staff of current efforts and activities in their respective regions.<br><br>The Wyoming SBDC considers training events an essential part of its public relations effort and an important service to provide clients information to build their business skills and improve their operations. For the program year, the SBDC Core Program, the CARES Act Program, PTAC and WSSI, held 119 training events with 2,509 attendees accounting for a total of 194 hours of training. All attendees are offered a survey at the conclusion of each training opportunity; the SBDC reviews client feedback and uses that data to inform the development and delivery of future training events. |

| *WBC Initiative* | Wyoming SBDC Supporting Activities and Execution |
|---|---|
| ***Investments and Services***<br>▪ *Revise programs to create investments with measured returns*<br>▪ *Develop services that clearly meet the needs of business, industry, and partners*<br>*Regularly review performance and adjust the portfolio and budget accordingly (cont.)* | **Develop services that clearly meet the needs of business, industry, and partners:**<br>As stated previously, post-training session and ongoing client surveys allow us to know how we are meeting client needs and what unmet needs we can address in the future.<br>The bulk of our client activity is individualized, one-on-one advising. As a result, our advisors build their skills and expertise in direct response to client needs and business trends. For example, having a robust and accurate online presence is vital for many small businesses; therefore, our advisors are resources for clients registering on Google My Business, and we provide training topics for enhancing online presence. Through experience, we also know that building a dynamic business plan is key for both startups and existing businesses seeking capital, but it is also a step many small businesses overlook or are hesitant to attempt on their own. Our staff not only has extensive expertise in various business model generation tools, including the Business Model Canvas, they utilize competitive intelligence market research and use that knowledge to assist startups in developing successful plans and existing businesses in revising current plans.<br><br>**Regularly review performance and adjust the portfolio and budget accordingly:**<br>Our efforts in this area were detailed in the explanation section above. Our state director constantly reviews budgets and expenditures, and where necessary and allowable, adjusts resources to meet emerging identified needs. On a more granular level, all staff, including the state director, experience an annual performance review. |
| ***Expertise***<br>▪ *Leverage industrial expertise to add value (to) core industries and activate new ones.*<br>▪ *Increase functional expertise in key areas of economic development, innovation, supply chain, startups, market development, workforce, finance, etc.* | The expertise of the Wyoming SBDC Network advising staff is varied, and includes assisting businesses in the technological, service, energy, recreation, and manufacturing sectors. One of our advisors is an international trade expert and in addition to the innovation resources present within our network through the WSSI program manager and others, we have a close relationship with Manufacturing Works and often refer and co-advise industrial clients with that partner.<br><br>Wyoming SBDC Network advising staff must complete a minimum of 40 hours of professional development annually, either in topics related to business advising or to their individual areas of business expertise. We are a learning organization and have deep expertise in |

| *WBC Initiative* | Wyoming SBDC Supporting Activities and Execution |
|---|---|
| ***Expertise***<br>▪ *Leverage industrial expertise to add value (to) core industries and activate new ones.*<br>▪ *Increase functional expertise in key areas of economic development, innovation, supply chain, startups, market development, workforce, finance, etc. (cont.)* | areas such as cybersecurity, digital marketing and e-commerce, government contracting, succession planning, international trade, etc. We are well-equipped to add value to businesses in identified core industries and assist businesses entering emerging markets.<br><br>**EXECUTION Examples:**<br>**Leverage industrial expertise to add value to core industries and activate new ones:**<br><br>Wyoming SBDC Network staff assisted many businesses operating in core and emerging sectors, including a craft distiller, manufacturer of high-end off-road camping trailers, meat processor, food processing operations, mobile veterinary service, and agricultural feed mill, just to name a few from our current success stories. Also, see our latest Federal Fiscal Year Annual Report for more impacts and success stories.<br><br>In addition to our core program efforts, the WSSI program manager actively works with technology-oriented companies in such areas as medical technology innovations, alternative energy efforts, and advanced optics development. In addition, PTAC staff currently assist small businesses to procure federal, state, or local government contracts in areas such as construction, parks and land management and maintenance, health care communication software, instructional technology and design, and real-time weather technology updates for snow removal.<br><br>**Increase functional expertise in key areas of economic development, innovation, supply chain, startups, market development, workforce, finance, etc.:**<br>The explanation in this section above details the areas of expertise SBDC advisors possess to address the needs of this point of the strategic initiative. Additionally, appropriate client referrals are made to Business Resource Network or other state resources for a holistic approach to client services. |

## WYOMING SBDC NETWORK PERFORMANCE DATA

| SBDC (including MRC) Counseling/Impact Only | | | |
|---|---|---|---|
| **WBC July 1, 2020 through June 30, 2021*** | **SBA Milestone Goal** | **Actual via Client Attribution** | **Percentage** |
| Clients Served | 340 | 1,765 | 683% |
| Capital Infusion | $9,008,459 | $13,188,040 | 146% |
| Business Startups | 26 | 40 | 154% |
| Jobs Supported** | 1,392 | 4,778 | 343% |
| Jobs Created/Retained | N/A | 104 | N/A |

*State FY results will not match Federal FY results and may vary over time since the database is not locked until the end of the Federal FY.
**Jobs Supported, required metric by SBA is derived from changes in session employee counts over all EDMIS (SBA database) time and requires at least two (2) client sessions for inclusion

| PTAC (including MRC) Counseling/Impact Only | | | |
|---|---|---|---|
| **WBC July 1, 2020 through June 30, 2021*** | **DLA Milestone Goal** | **Actual** | **Percentage** |
| Clients Served | 144 | 289 | 201% |
| Counseling Hours | 925 | 736** | 80% |
| Participated or Sponsored Events | 25 | 31 | 124% |
| Contracts | N/A | $59,749,685*** | N/A |

*State FY results will not match Federal FY results and may vary over time since the database is not locked until the end of the program's Federal FY.
**We have been short a procurement specialist since April 5, 2021. The replacement will begin Sept. 1, 2021.
***466 awarded contracts make up this total.

| MRC Activity | | | |
|---|---|---|---|
| **WBC July 1, 2020 through June 30, 2021** | **MRC Milestone Goal** | **Actual** | **Percentage** |
| Number of Projects | 315 | 328 | 104% |

## SBDC NETWORK PREFORMANCE DATA (cont.)

| Client/Non-client Contact Time by Program | | | | |
|---|---|---|---|---|
| **WBC July 1, 2020 through June 30, 2021*** | **Client Count (Distinct)** | **Session Count** | **Contact and Prep Hours Sum** | **Non-client Information Transactions*** Recorded Hours** |
| **SBDC**** | 1,765 | 4,945 | 3,971 | 302 |
| **PTAC** | 289 | 1,336 | 736 | 50 |
| **WSSI/FAST** | 62 | 481 | 365 | 37 |
| **PA1605****** | 6 | 8 | 14 | 11 |
| **CARES Act** | 226 | 650 | 581 | 3 |
| **Network Total** | 2,348 | 7,420 | 5,667 | 403 |

*State FY results will not match Federal FY results and may vary over time since the database is not locked until the end of each program's Federal FY. Unless otherwise noted, data extracted from July 1, 2020 through June 30, 2021.

**SBDC includes the market research hours of the MRC team (not including Information Transfers)

***Labeled and tracked as Information Transfers = dissemination of information to individuals who are not registered as clients. Information Transfers does not include reportable impact other than time.

****PA1605 is the SBA Portable Assistance program requiring No Cash Match. Data for PA1605 is run on its program period of May 1, 2021 through April 30, 2022 or for this reporting, May 1-June 30, 2021. Assistance focused on businesses affected by the economic downturn excluding the pandemic.

| Events by Program | | | | |
|---|---|---|---|---|
| **WBC July 1, 2020 through June 30, 2021*** | **Event Milestone Goal** | **Actual** | **Number of Attendees** | **Classroom Hours** |
| **SBDC**** | N/A | 13 | 404 | 15 |
| **PTAC** | 25 | 31 | 495 | 51 |
| **WSSI/FAST** | 11 | 13 | 246 | 56 |
| **PA1605***** | N/A | 0 | 0 | 0 |
| **CARES Act** | N/A | 62 | 1,364 | 72 |
| **Network Total** | 36 | 119 | 2,509 | 194 |

* State FY results will not match Federal FY results and may vary over time since the database is not locked until the end of each program's Federal FY. Unless otherwise noted, data extracted from July 1, 2020 through June 30, 2021.

**SBDC includes the market research hours of the MRC team (not including Information Transfers)

***PA1605 is the SBA Portable Assistance program requiring NO Cash Match. Data for PA1605 is run on its program period of May 1, 2021 through April 30, 2022 or for this reporting, May 1-June 30, 2021. Assistance is focused on businesses affected by the economic downturn excluding the pandemic.

# The WBC-State Economic Development Strategy Alignment – Technology Transfer-Research Products Center

***WBC Purpose*** *– Creating new opportunities for current and future generations of Wyomingites by ADDING VALUE to Wyoming's core industries and LEVERAGING them to ACTIVATE new economic sectors*

**Technology Transfer-Research Products Center Purpose -** During the contract period we provided patent and trademark research for Wyoming independent inventors and providing general information on intellectual property issues related to their potential business endeavors, coupled to the University of Wyoming's Land-Grant University charter of serving the state. As the Technology Transfer Office for the University of Wyoming, Technology Transfer-Research Products Center works closely with University of Wyoming inventors to evaluate, protect, and out-license University of Wyoming technologies in various cutting-edge fields of research that have the potential to expand the economic sectors in the State of Wyoming as well as supporting and strengthening the already established core industries of Wyoming.

**Technology Transfer-Research Products Center Mission -** By assisting Wyoming independent inventors and entrepreneurs in providing early-stage non-legal assessment of their inventions or proposed trademarks, Technology Transfer-Research Products Center helped them to make more informed decisions about whether to invest their time and money in pursuing formal protection of intellectual property through patent filings or trademark registrations. Also, because these inventors are more aware of where their technology sits within the patent landscape, they are able to more quickly redirect efforts to other pursuits that may more likely benefit the state economy.

***WBC Vision*** *– Realize diverse, broad and lasting growth so that Wyoming can prosper no matter the economic climate or status of individual sectors*

**Technology Transfer-Research Products Center Vision -** The Wyoming Technology Transfer-Research Products Center is dedicated to evaluating, protecting, and promoting technologies in all areas of research from cutting edge fields to core industries so that the research coming out of the University of Wyoming is available to benefit key stakeholders at the University of Wyoming and across the State of Wyoming.

| *WBC Initiative* | Technology Transfer-Research Products Center Supporting Activities |
|---|---|
| ***Add Value to Core Industries*** *natural resources, tourism and outdoor recreation, agriculture* | Technology Transfer-Research Products Center has worked with Wyoming Independent Inventors in all industries who have requested assistance. |

| *WBC Initiative* | Technology Transfer-Research Products Center Supporting Activities |
|---|---|
| ***Add Value to Core Industries*** *natural resources, tourism and outdoor recreation, agriculture (cont.)* | Technology Transfer-Research Products Center supports all fields of research at the University of Wyoming and has a strong history of working with University of Wyoming Inventors in natural resources and agriculture. |
| ***Activate New Economic Sectors*** *healthcare; financial, scientific, and professional services; digital and technology; arts and culture; advanced manufacturing* | Technology Transfer-Research Products Center has worked with both the NIH-funded ASCEND Western Hub which focuses on strengthening and expanding bio health technologies and companies coming out of university research.<br><br>Additionally, work has been done with the SPARK bio hub based in the College of Health. Reviews and assistance with numerous health-focused technologies coming out University of Wyoming research have also been completed.<br><br>Technology Transfer-Research Products Center regularly reviews and works to protect technologies stemming from University of Wyoming research focused on software-based technologies in a number of fields from healthcare, natural resources and economic strategies. |
| ***Local Economic Development***<br>▪ *Foster a culture of regional connectedness*<br>▪ *Empower communities to sustainably develop their economies*<br>▪ *Work with all partners to develop effective approaches to Economic Development* | Technology Transfer-Research Products Center connects regularly with our BRN colleagues from IMPACT 307, the Wyoming SBDC Network, and others. Assistance was provided directly, during the time when the Transfer Office was supported by the WBC and was performing outreach services.<br><br>Assistance is now provided through work with University of Wyoming spinout companies that license University of Wyoming technologies along with assisting more generally and providing support to the other BRN entities now that the Transfer Office is no longer supported by the WBC.<br><br>The Technology Transfer-Research Products Center regularly directs University of Wyoming and Wyoming residents to resources that are available to assist them with moving forward with their business ideas.<br><br>The Technology Transfer-Research Products Center continues to work closely with colleagues within the University of Wyoming and around the State of Wyoming to support economic development initiatives by providing input on intellectual property best practices and information on University of Wyoming technologies that may be applicable and available for licensing. |

| *WBC Initiative* | Technology Transfer-Research Products Center Supporting Activities |
|---|---|
| ***Partnerships***<br>▪ *Coordinate inter-agency plans and programming*<br>▪ *Align advisory boards*<br>▪ *Increase communication across partnerships*<br>▪ *Increase alignment and clarity of roles and responsibilities.* | The staff at the Technology Transfer-Research Products Center works regularly with IMPACT 307 to offer educational assistance to University Of Wyoming and the other BRN entities on intellectual property and best practices for management thereof.<br><br>Numerous discussions have been held about what support services the Technology Transfer-Research Products Center can appropriately provide as the University Of Wyoming Technology Transfer Office and the scenarios in which involvement is appropriate. |
| ***Investments and Services***<br>▪ *Revise programs to create investments with measured returns*<br>▪ *Develop services that clearly meet the needs of business, industry, and partners*<br>▪ *Regularly review performance and adjust the portfolio and budget accordingly.* | Due to the large amount of time and resources the Technology Transfer-Research Products Center invested in the Independent Inventor Program, a cost-benefit analysis of the Program as they applied to the individual independent inventors, the Technology Transfer Office and stakeholders was necessary. The analysis indicated that the cost of administering the Independent Inventor Program did not justify offering the program.<br><br>Only a handful of the independent inventors that were assisted by the Technology Transfer-Research Products Center, ultimately pursued and successfully obtained patent protection and even less were able to capitalize on their patents as a basis for successful business endeavors.<br><br>It was thus decided that the efforts of the Technology Transfer-Research Products Center staff and the funding involved were better used when focused on University of Wyoming intellectual property, spinouts, and partnerships with third parties, rather than trying to perform limited services for those outside of the University of Wyoming.<br><br>**EXECUTION Examples:**<br>The Independent Inventor cost-benefit analysis found that the Technology Transfer-Research Products Center assisted 65 independent inventors/entrepreneurs between July 2019 and June 2021. Three to five hours of staff time was recorded per inventor for research and reporting of their proposed patents. A $3000 reservation fee was also paid to a patent attorney from the Hogan & Lovell IP firm for consultations with Independent Inventors in January 2020. Only one inventor chose to meet before the engagement was discontinued in 2021. The Technology Transfer-Research Products Center was never able to confirm that the inventor moved forward with the application. |

| *WBC Initiative* | Technology Transfer-Research Products Center Supporting Activities |
|---|---|
| ***Expertise***<br>▪ *Leverage industrial expertise to add value (to) core industries and activate new ones.*<br>▪ *Increase functional expertise in key area of economic development, innovation, supply chain, startups,*<br>▪ *market development, workforce, finance, etc.* | The Technology Transfer-Research Products Center is directed by a licensed patent attorney with undergraduate and graduate degrees in a technical field, and is regularly undertaking training to stay up to date on the latest trends both in technology transfer practices, legal issues, as well as business trends.<br><br>The Technology Transfer-Research Products Center consults and engages with various patent attorneys' outside of the University of Wyoming on more complex patent applications in both the United States and other countries. |

# Appendix A
# Client Testimonials

## IMPACT 307

### LARAMIE

### ALTUS – ENGAGE TOGETHER

"Altus Solutions, Inc. is a business for good that exists to build higher solutions that reach greater impact in the fight end human trafficking and protect the vulnerable in four primary areas – tech, education, finance, and business. Headquartered in Laramie, Wyoming at the WTBC, we are presently recruiting both a local and national team of human rights lawyers, business developers, technologists, educators, community consultants, marketers, impact analysts, and more.

I had been dreaming of launching a business for good that would help accelerate the anti-trafficking movement for some time. But having spent nearly two decades in the nonprofit sector, I knew I needed a great deal of coaching and help to get it done. We met with the amazing leadership team at WTBC in March, and became a client in April 2019. WTBC has been the accelerator for us to make our dream of creating a business to accelerate the end of human trafficking a reality. Their coaching, advice, expertise, accessibility, support, network, and daily guidance and encouragement helped us move from concept to launch in just seven months' time. We are now in the investment raising phase of the company, and the WTBC team continues to help us make it all happen. I could not have done it without them."

***Ashleigh Chapman, JD, CEO of ALTUS***

### CODEUS TECH

"IMPACT 307 has been a critical part of Codeus Tech's success as well as my (Cody Fagley) personal career development. When I first entered their office in summer of 2017, I had a neat idea but no skills necessary to execute it. I had very little experience in technical writing or public speaking outside of university classwork. These skills were refined over the last four years under the mentorship of IMPACT 307's team and other incubated companies. Although the entrepreneurs are all at different points in our careers, we are able to come together and help each other through mutual struggles and develop relationships as though we were all varying wings of one organization.

The benefit of IMPACT 307's help is far beyond what it has done for my company's growth. Although I did not see any direct monetary success through my four and half year journey, I am much further in my career by any other metric than if I had taken a "traditional" career path. Since entering (public) hiatus one month ago to pursue my PhD in cyber-security at UW, I have been given more and better opportunities than I would have been given otherwise. These opportunities are being presented by other companies in the incubator, IMPACT 307 staff, as well as the main campus of the University of Wyoming."

***Cody Fagley, CEO and Founder of CODEUS***

### CHEYENNE

### CRAVE NUTRITION

"In the fall of 2019, I was a Registered Dietitian that ever-so-technically became a business owner when I opened a private practice in Cheyenne. I had a vision for how I could help Wyomingites' health through

nutrition, but I lacked the business know-how to turn that into reality. I learned about Impact 307 when they hosted an informational session at the Laramie County Library about the Southeast Wyoming Innovation Launchpad (SEWYIL) startup challenge. After applying, I was thrilled to find out I was selected as a finalist.

Thanks to SEWYIL, I, alongside a cohort of peers, got a crash course in all things business. Guest lectures and Q and A sessions from community partners, including accountants, lawyers, bankers, and a marketing team, as well as one-on-one help to wrap my head around my customer market and cash flows, all significantly increased my confidence as a business owner. The challenge culminated in a pitch night--something I had NEVER participated in before--and let me tell you, sharing my vision and planning for my business to a crowd of community members is one of the most empowering and motivating experiences I've ever had. The absolute icing on the cake was being awarded grant funding from Impact 307 that night.

Amazingly, the support didn't end there. Thanks to ongoing mentorship, I've been able to make connections with a larger network of partners and I've gotten help problem solving through challenges that have come up since participating in SEWYIL. It's been less than a year since opening my practice, but thanks to Impact 307 I've been able to steadily build a profitable business that is not only creating economic opportunities but is truly changing the lives of the clients that have sought out services to better manage their health."

***Lindsey McCoy, CEO and Founder of Crave Nutrition***

## SHERIDAN

### WOLF'S COSPLAY

"The Sheridan Startup Challenge was instrumental in helping my business, Wolf's Cosplay, go from a simple side business for extra cash to a full-fledged business with employees. With the support and counsel of the organization and judges I was able to effectively create and implement a business plan that allowed me to maximize the growth potential of my business. Additionally, with the help of all involved, I was able to understand what I was missing in my plan and make adjustments to avoid pitfalls. The combined business experience of the group was invaluable to me, and I was able to make professional contacts I would have not been able to otherwise. An example of this is my provided membership to the Phorge Makerspace and access to their laser cutting equipment. That access alone has enabled me to add a dozen products to my catalog.

In addition to the guidance the Startup Challenge provided, the financial aspect of their support was significant. Being awarded over $30,000 is no small thing. That is a life changing amount of money. With those funds I was able to purchase much needed equipment to ramp up my operations from a small studio to a full-scale manufacturing facility. I would have been able to do this over time myself, but by injecting the funds into my business I was able to get a jump on 2021 and close a number of contracts I would not have been able to otherwise. The equipment purchases also set the stage for being able to hire additional employees and provide them with a professional and safe work environment.

Without a doubt the Sheridan Startup Challenge has changed my life and added a new and unique business to Sheridan. What Scot and his team have done are amazing and should be supported. I know that in the coming years I will look for every opportunity to support the program myself and those who participate."

***Jason Hodges, CEO Wolf's Cosplay***

**CASPER**

**DISA USA**

"Impact 307 (formerly WTBC) has provided invaluable support and services for our startup company, DISA. We have now been in the incubator for roughly 3.5 years and taken our startup from an idea to a business with four full time employees, six part time employees and are now about to close on our second fundraise.

The building has been fantastic to base our company from where we have high quality, affordable, office space. Recently, a valuable addition has been being located in an opportunity zone. One of our current investors exclusively invests in new technologies located in opportunity zones. The location of the incubator has allowed us to receive support from this group.

Regarding mentorship, the entire team has been great for guidance, practice pitches, and overall strategy. Anytime we need something we can go to Eric and John and receive their advice and ideas. Furthermore, the team has provided great connections and networking opportunities.

We are thankful for all the support from Impact 307 and look forward to our future here!"

***John Lee, DISA USA***

## Manufacturing-Works

**STRAIGHT JACKET ARMORY**

"It's great to work with Manufacturing Works-Wyoming, an organization that took time to first find us and seek us out and then learn about our business and understand our culture to better support our requirements. This is our first exposure to Manufacturing Works consulting, and we will continue to use them as a force multiplier that produces intuitively positive solutions to challenging problems that produce mission success. Today, my company is growing, much stronger and works better as a team. The value that Manufacturing Works and their consultants provided is highly valued and as such we continue to work with them. This is a tremendous asset for the state, especially for the rural small manufacturers."

*Results: 2 new jobs created, 50% increase in contract backlog - approximately $450- 550,000, 50% increase in annual revenue, $300,000 increase in annual revenue due to expansion into new markets.*

***Tisten Arnold, Co-Owner***

**PUCKER FACTOR PRECISION**

"It's great to work with Manufacturing Works-Wyoming, an organization that took time to first find us and seek us out and then learn about our business and understand our culture to better support our requirements. This is our first exposure to Manufacturing Works consulting, and we will continue to use them as a force multiplier that produces intuitively positive solutions to challenging problems that produce mission success. Today, my company is growing, much stronger and works better as a team. The value that Manufacturing Works and their consultants provided is highly valued and as such we continue to work with them."

*Results: Contract backlog increased by 100% ~$250-300K, ventured into new markets increasing revenue stream by $200K per annum, expanded backlog allowed us to add 2 full time employees, retained all current customers and expanded customer sale volume by over 50%.*

***Brian Woodward, President/Owner***

**LUCID UVC INC**

"MW took part in the community service cleaning of the Comea Shelter to build awareness of the products. This awareness paved the way for local government and business sales in the city of Cheyenne and was featured on local TV."

Results: *83% of current sales were because of Manufacturing Works help. 66% of units sold because of Manufacturing Work network help in the 1st 6 months. Manufacturing Works sought out and confirmed several manufacturing capabilities for full product deployment.*

***John Edwards, Owner/CEO***

# Wyoming SBDC Network

## Attributed Client Testimonials

**Superior Paddlesports**

"Rob and Mike from the SBDC have helped me navigate my business startup process including great market research. As a new business owner I had a lot of questions about the process including legal concerns, permitting, licenses, website/SEO work, advertising, etc. My advisors at the SBDC gave me valuable, timely support and invested themselves in my success. I am extremely grateful for the SBDC and the free service they offer to businesses in Wyoming!"

***Frazer Tear, Superior Paddlesports***

**USA Credit Card Processing with Maverick Bank Card**

I couldn't be happier with the variety of help I received from SBDC Network. They listened to me explain my company and how I wanted to tackle my sales and marketing, then they offered great ideas and challenged me with doing things I wouldn't have tried on my own. They provided me the financial means to take it to the next level as well as gave me six hours of professional help in the area of expertise that I lacked knowledge in. I would highly recommend SBDC to any business who wants to grow their company no matter how small or how big. They are truly here to help. Thank you.

***Carolea Radke, USA Credit Card Processing with Maverick Bank Card***

**WyoNaturals**

When the Wyoming SBDC Network starting helping us, we were at a loss on how to proceed where our business was concerned. Since we started working with the SBDC, we had a direction again, and we really appreciate the work that you've done.

***Brian Henson, WyoNaturals***

**Client Feedback from Periodic Surveys**

"With the different bills, restrictions and categories; the whole "virus" situation was a little hard for a lay person that meanwhile had to run their business to keep track of all the options, clauses, requirements. My bankers did not do a good job of explaining the program, in my opinion. Talking to John worked well and I knew how to proceed. Thanks!"

"I am incredibly grateful to have been introduced to the SBDC Network—this is an incredibly valuable resource not just for my small business, but for countless businesses across the State of Wyoming. We are incredibly fortunate to have this incredible organization available to us—thank you!"

"I can't believe this service is free! What an amazing resource for budding and established entrepreneurs!"

"I am impressed with the promptness of the replies and the follow-up correspondence. I am also impressed with the flexibility to meet in a place that is convenient to me and not just the advisor. I appreciated that Steen did not make me feel like I was a complete moron when trying to figure out my next steps. Thank you!"

"I appreciated the honesty and help he was able to give me. I knew that he didn't have any other motive than to help my business succeed."

"I have already referred others to this service. It is a valuable resource!"

"I started with some basic needs for shoring up the foundational structures of my business . . . the resources available are well beyond what I had expected and are helping to take my business development to another level."

"It's been truly wonderful to have the guidance of the SBDC throughout my startup, my product development, and now the scaling process. Thanks for all you do!"

## 3. Priority #4- Office of Research and Economic Development put together specific request for a test module for 2 years of funding so you could pick the lowest hanging fruit to build this together

**University of Wyoming - Office of Research and Economic Development**
**FY23-24 Supplemental Budget Request**
**Justification and Funding Priorities**
**January 7, 2022**
**(update of the August 5, 2021 version)**

**Background:**

The mission of the University of Wyoming (UW) Office of Research and Economic Development (ORED) is to work with faculty, staff and students in all of UW's colleges and outreach programs to link research, technology transfer, and economic development efforts to enhance federal, state and private sector support for faculty and graduate student scholarship. As the state of Wyoming addresses budget concerns and traditional Wyoming industries face economic pressures, it is imperative that the state's only four-year degree granting institution be on the forefront of responding to the driving forces behind research and economic change. UW needs to provide students with the education and skills they need to compete for employment in the technical and digital world as well as hire faculty who perform cutting edge research in scientific, technological, social science, and humanity fields.

**Request:**

Research faculty in all disciplines require a certain level of central administrative staffing to assist with the complex regulations and policies associated with grant funding. As evidenced through external evaluation (Attain and Point) and comparison to other universities of similar size, UW is woefully understaffed in all areas of research administration. As such, UW seeks $1 million in supplemental re-occurring funding to support 12 hires in critical areas of research administration and provide service to campus and support state investments in research infrastructure.

Approval of this supplemental funding request will allow UW to enhance research productivity. If UW wants to be competitive with other institutions to attract talented research faculty, prospective hires will go where they see they have the institutional support they need to be successful. Hiring of talented faculty results in the ability to attract high caliber graduate and undergraduate students. Provision of foundational administrative staffing and support is the launchpad to increasing UW's ability to recruit talented faculty and students and to show research funding agencies UW has the means to support their research and economic development efforts.

**Justification:**

Universities hire research administrators to help scientists apply for grants, ensure scientists comply with institutional policies and federal laws, and coordinate research with national and international collaborators. It can be difficult for busy scientists to keep up with constantly shifting regulations and protocols -particularly with respect to grants - and administrators ensure that faculty researchers do not run foul of any rule changes. Research administrators take care of the necessary tasks that researchers often cannot do - and should not do - so scientists can be left to focus on their research and scholarly

activities. The research enterprise cannot grow without adequate staff resources to support basic services for campus and ensure research compliance.

In addition to research administration staff, universities require technical staff to manage and operate highly technical research facilities and instrumentation. The State of Wyoming has made substantial investments in research facilities and instrumentation in recent years. Without the proper staffing to manage the buildings and the highly technical instrumentation contained in the buildings, the investment is wasted.

UW is currently classified as R2 – "higher research activity" in the Carnegie classification for doctoral granting institutions. As UW aspires to reach the Carnegie R1 "highest research activity" classification for universities, as well as achieve Tier 1 status in engineering, UW must substantially increase staffing in the areas of pre-award proposal development, technology transfer, and shared research facility management. Additional staffing in all of these areas is imperative to establish a foundation of research support for all UW researchers and for adequately preparing students for the workforce.

The ORED staffing shortage was confirmed through a November 2020 study conducted by an outside consulting firm (Attain). UW hired Attain to write a request for proposals (RFP) to solicit vendors of electronic research administration systems. To determine the requirements for the RFP, Attain conducted a series of interviews with ORED pre-award, compliance, and technology transfer staff; Office of Sponsored Programs (OSP) post-award staff; department business managers; and faculty researchers. The interviews asked for strengths and weaknesses in campus research services. The overwhelming weakness voiced in the interviews was that ORED is sorely understaffed. As a comparison, other R2 universities with similar annual research expenditures to UW (Boise State University and University of Idaho) have double the number of pre-award and compliance staff as UW.

**Funding Allocation:**

In January 2021, President Seidel created a campus working group to revise UW Regulation 9-2: Indirect Costs Policy and provide recommendations for a proposed new distribution of indirect costs at the University of Wyoming. The working group examined the broader questions of how to: (1) promote and enhance research at UW; and (2) determine how indirect funds from sponsored programs can be best used to maintain and grow UW's research enterprise. To accomplish this, the working group developed a spreadsheet titled, *Baseline Services Needed to sustain and Build UW's Research Enterprise and Meet Compliance and Contractual Obligations.*

The working group reviewed staffing of peer institutions and determined what positions at UW were needed to ensure compliance with federal, state, local, and UW regulations and policies; bolster staffing to avoid overwork and burnout of current staff; and meet a legislative or contractual obligation. The working group created a spreadsheet listing needed positions and categorized them based on the criteria listed above. The top priority hires met all criteria - compliance insurance, reduce workload of current staff, and meet a contractual obligation.

The working group identified over 40 positions needed to enhance research activity at UW. Eight of the top priority positions were included in the FY22 budget and have been filled. These critical service positions have increased ORED's salary costs by $1 million/yr, or an increase of 10%. An additional $1

million of recurring supplemental funding will allow ORED to continue make the following additional critical hires that currently cannot be supported.

An additional $1 million would allow ORED to hire 12 positions, each described below. The position priority, cost of these positions (based on current salaries for similar positions) are listed in Table 1.

**Priority 1: College Res Coordinators (4)** – Pre-award assistance coordinators (four of them) would be placed in the colleges/schools that are the most research intensive. This includes the College of Agriculture and Life Sciences, College of Engineering and Physical Sciences, College of Health Sciences, and one coordinator shared by the School of Energy Resources/Haub School/Humanities, Social Sciences, Arts College. These positions would assist faculty in those colleges with preparation of research funding proposals so that faculty can focus on writing and developing substantive proposal content. Providing faculty with proposal preparation assistance allows them to produce more competitive proposals, thereby improving their chances for funding. College Research Coordinators are commonplace in most R1 research universities as they take the administrative duties of proposal development off the researcher so they can focus on substance. ORED would request that each college or units sharing the coordinator contribute to 50% of the salary cost of the position. The four Coordinators would report to the ORED Director of Research Services.

**Priority 2: Technology Transfer Office (TTO) – Business Development Associate** – Currently, the TTO has 3 full-time staff – A Director, Licensing Manager, and a Technology Manager. The Business Development Associate would report to the Director and conduct initial assessment of commercial potential of new technologies, develop marketing materials, evaluate market size, identify potential licensees and key contacts, make initial marketing contact with potential licensees, maintain industry contacts and assist other TTO personnel with case management, with a focus on the marketing aspects of each case. This position would work with researchers to encourage engagement with the commercial sector to generate interest in new technologies developed at UW.

**Priority 3: Technology Transfer Office (TTO) – Office Associate –** With the TTO having the specialized positions of Director, Licensing Manager, Technology Manager, and a future Business Development Associate, an Office Associate is needed to manage the day-to-day business affairs of the office. This position would report to the Director of TTO. At this time, the specialized positions are having to take on these duties, which takes away from the specialized duties they were hired to do.

**Priority 4: Research Compliance – Animal Research Facility Manager –** UW has numerous animal research facilities scattered around campus. The number of animal research subjects will increase when the new vivarium at the Science Initiative Building opens in the spring of 2022. There are other new faculty at UW who intend to significantly increase large and small animal research in areas of particular importance to the agricultural industry in Wyoming. UW does not have a central position to oversee the practices and procedures taking place in these various animal research facilities. Lack of oversight to ensure consistent practices and adherence to federal laws on animal care makes UW vulnerable to findings during federal inspections. An animal research facility manager is needed to be responsible for the day-to-day supervision and overall management of laboratory animal care personnel and the animal facility. This is a position commonly found at R1 research institutions. A consistent clean compliance record with federal regulations gives sponsors confidence in providing funding to research institutions.

**Priority 5: SI – CASI Manager** – The SI building will house the Center for Advanced Scientific Imaging (CASI). This facility will contain millions of dollars of highly specialized imaging equipment to be shared among research faculty and students. A specialized technician is needed to oversee use, maintenance, and repair of the instrumentation to ensure protection of the significant investment. The CASI will be a shared instrumental facility for the UW campus and will also serve clients external to UW. Acquisition of the state-of-the-art instrumentation slated for the CASI will enable UW researchers to take their work to the next level. Also, sponsors are more inclined to provide funding to institutions that can demonstrate they have on-site access to advanced instrumentation and the expertise in-house to run and maintain that instrumentation.

**Priority 6: SI - Greenhouse Manager** – a specialized technician is required to manage the greenhouse space and research/educational activities in the SI building. As with the CASI, having on-site access to dedicated greenhouse space that is well maintained and operated, makes UW an attractive investment for research dollars. This position would report to the SI Building Manager and is a top priority hire.

**Priority 7: SI – Program and Building Manager** – The new Science Initiative (SI) building is slated to open in the spring of 2022. A building manager is necessary for oversight of activities in the building, managing building visitation, ensure maintenance and repair of the building, facilities, and equipment within the building. This is a high priority hire and should be hired prior to occupancy of the SI building. This position would report to the Science Initiative lead within ORED.

**Priority 8: Genome Tech Lab Manager** – The Genome Technology Lab is a new facility created with funding by the current National Science Foundation (NSF) EPSCoR grant ($20 million over 5 years). As a condition of that funding, UW is obligated to sustain this lab for the long term. A laboratory technician position is currently funded from the NSF grant but will need to be funded once the grant ends in the summer of FY22. This position will report to the ORED Associate Vice President for Research.

**EcoBGC Lab Manager** - The Ecobiogeochemistry Lab is a new facility created with funding by the National Science Foundation(NSF) EPSCoR ($20 million over 5 years). As a condition of that funding, UW is obligated to sustain this lab for the long term. A laboratory technician position is currently funded from the NSF grant but will need to be funded once the grant ends in the summer of FY22. This position will report to the ORED Associate Vice President for Research.

**Table 1: Estimated Cost of Requested Hires**

| | **Positions, in Priority Order** | **# of FTEs** | **Base Salary** | **Salary+Fringe (49.9%)** |
|---|---|---|---|---|
| 1 | College Research Coordinators - It is anticipated that the colleges/units will cover ½ of the salary of each position. ORED will pay the other ½. The cost shown here is the cost to ORED for 4 Coordinators. | 4 | $45,000 ea, ORED pays ½ = $90,000 | $134,910.00 |
| 2 | Technology Transfer Office (TTO) – Business Development Assoc. | 1 | $75,000.00 | $112,425.00 |
| 3 | Technology Transfer Office (TTO) – Office Assoc. | 1 | $45,000.00 | $67,455.00 |
| 4 | Research Compliance – Animal Research Facility Manager | 1 | $75,000.00 | $112,425.00 |
| 5 | SI – Program and Building Manager | 1 | $90,000.00 | $134,910.00 |
| 6 | SI - Greenhouse Manager | 1 | $65,000.00 | $97,435.00 |
| 7 | SI – CASI Manager | 1 | $75,000.00 | $112,425.00 |
| 8 | Genome Tech Lab Manager and EcoBGC Lab Manager | 2 | $136,000.00 | $203,864.00 |
| | **Totals** | **12** | **$651,000.00** | **$975,849.00** |

**Funding Outcomes:**

Currently, UW is classified as an R2 “higher research activity” in the Carnegie classification for doctoral granting institutions. R-1 indicates “highest research activity,” R-2 “higher research activity,” and R-3 “moderate research activity.” To be classified as an R-1 doctoral university, an institution must award at least 20 research-based doctoral degrees per year. R-1 research productivity is measured by the number of research doctorates awarded plus research staff, and the amount of research expenditures, scaled to the number of faculty.

Achievement of R1 status requires an institution to provide robust support from central administration for academics and research. As stated above, UW is woefully understaffed in the area of research administration compared to other peer universities. Our trajectory toward a Carnegie R1 designation must start with providing the staffing resources faculty need to focus on their research, attract talented graduate students, and be successful at obtaining and keeping external grant funding.

Research faculty also depend on research buildings, infrastructure, and instrumentation to conduct their research. Large investments made in buildings such as the Science Initiative Building require management and technical staff to keep the facilities in working order and work with faculty, staff and students to monitor access to shared space and instrumentation. A building manager, greenhouse manager and instrumentation center manager are needed to protect the state of Wyoming’s substantial and generous investment in the Science Initiative Building.

Approval of this supplemental funding request will allow UW to greatly improve research administrative services to campus and result in greater research productivity. Competition for grant funding is more intense than ever and UW needs to position itself to be competitive with other institutions that have the staffing they need to make their faculty successful.

This additional financial investment in ORED will be an attractant to candidates for a permanent VPR.

## 4. Priority #7 College of Agriculture and Natural Resources- Asked UW to provide in writing the breakdown of the 7 positions requested by the College of Agriculture

| Title | Salary | Position Description |
|---|---|---|
| Asst or Assoc Prof., Plant Sciences/UWE | $81,421 | EXCEPTION REQUEST: The importance of forage production ($400M annual value) is felt by nearly every agricultural operation in the state of Wyoming. Invasive species impact a vast majority of grazed and hay production acres across the state and region, causing tremendous economic losses and environmental degradation. This position will improve and broaden our support of this critical sector of the agricultural economy. The faculty line will contribute to two major strategic programs, IMAGINE (Invasive Plants) and RMAL (Ranch Management & Ag Leadership) in addition to other departmental, college, and university goals. |
| Asst Prof, Advanced Technologies in Plant Protection & Crop Science | $85,000 | EXCEPTION REQUEST: Agriculture is currently undergoing a digital revolution. Autonomous robotic planters and weeders, precision nutrient and pesticide application equipment, aerial crop scouting with satellites and UAVs, harvest yield monitors, and other current agricultural technologies are becoming commonplace on farms across the country. Vast amounts of data are being collected and analyzed to improve crop production efficiency and to lower the environmental footprint. As a result of the rapid changes in the crop production industry, employers are looking for graduates with high-tech skills – programming, data science, remote sensing, GIS and others. |
| Assoc/Full Prof | 135,000 | EXCEPTION REQUEST: The Asst. Director of the Agricultural Experiment Station manages the internal operations of the research enterprise of the College, freeing the Associate Dean and Director of AES to focus on engagement with external stakeholders, major grants, infrastructure projects, and national programs. |
| Asst Prof/Specialist, Rangeland Ecology | 85,000 | EXCEPTION REQUEST: The candidate will help build the teaching and research expertise in integrated rangeland systems and lead outreach and engagement focused on ranch and range operations in the state of Wyoming. The candidate will work in intensively managed grazing lands and be able to educate students on effective and appropriate management of grazing lands so that students will be well trained for both private and pubic sector jobs that require an understanding of both ecology and management. Wyoming has a strong tradition of working closely with ranchers and allied agencies and industries to tackle complex production and environmental problems. This has included rangeland improvements to reduce invasive plants, water quality on public grazing allotments, animal performance at high elevations, grazing management, etc. At present we are unable to fully meet the needs of the state in ranch and rangeland operations, and this position will fill a critical gap in statewide stakeholder engagement. |
| Academic Professional, UWE Enterprise Development Specialist, Food and Nutrition | $81,025 | EXCEPTION REQUEST: Position will expand the enterprises of Food, health, and nutrition critical for the vitality and health of rural communities, specifically the ability of our children and families to have sufficient healthy food, highlighted by the First Lady's Hunger Initiative. This position will be outcome-focused, stakeholder responsive, Wyoming relevant, interdisciplinary, and empowered to innovate through collaboration with faculty on campus and others. |
| Asst Pro. Bioinformatics, Comp Biol. | $78,127 | EXCEPTION REQUEST: Assistant Lecturer position increases the effectiveness of instruction computational biology across a diverse array of undergraduate courses that would address the need of students in biological science across campus to become competent in this area. Lecturer would lead development of an on-line MS and post-baccalaureate certificate program in Computation Biology. Existing university faculty that have competency in a wide-variety of computational biology applications – bioinformatics, modeling, generation and analysis of large data sets – and are among the most productive on campus, but are busy with core instructional assignments, managing funded research programs, and are inordinately tapped to serve on University committees. |
| Asst Prof, Nutrition | $79,000 | EXCEPTION REQUEST: The Didactic Program in Nutrition and Dietetics (DPND) is an accredited program through the Accreditation Council for Education in Nutrition & Dietetics (ACEND). Students cannot become a Registered Dietitian Nutritionist (RDN) without graduating from an accredited program. As of 2024 students will be required to hold a master's degree before they can sit for the RDN credentialing exam. In order to remain competitive with peer institutions and attract quality students, as well as be ready to produce students who can sit for the RDN exam, the UW DPND program must move away from an undergraduate DPND program and toward a 4+1 master's degree program. However, to move to this 4+1 program, we require another tenure track faculty member. |

5. **Unit 6708 Brucellosis Testing Research- Do we do research with other universities on Brucellosis? Do we share the data with other states?**

The brucellosis test data goes to the state veterinarian and to USDA APHIS. They control the data but it is not secret and is shared at the US Animal Health Association meeting. The state needs to be, and is, transparent about our test numbers as this is essential to maintaining our credibility with trading partners. We also hold an annual meeting of the Governor's Brucellosis Coordinating Team, chaired by Will Laegreid with support from Dr. Bruce Hoar (Vet Sci and COANR), which is attended by both Montana and Idaho representatives involved in testing and numbers are certainly discussed among other topics.

In terms of research, the University does a fair bit of brucellosis research. Currently our funded researchers include Brant Schumaker, Liz Case and Patrick Johnson. These projects involve molecular epidemiology, improved test development, genomic sequencing, etc.

Appendix A provides some of the recent publications:

1. Ecological Applications, 30(6), 2020, e02129- Ecological Society of America: Chronic wasting disease undermines efforts to control the spread of brucellosis in the Greater Yellowstone Ecosystem: Matthew Maloney, Jerod A. Merkle, David Aadland, Dannele Peck, Richard D. Horan, Kevin L. Monteith, Thach Winslow, Jim Logan, David Finnoff, Charles Sims and Brant Schumaker

2. Journal of Veterinary Diagnostic Investigation 2020, Vol. 32 (5) 700-705: Comparison of 2 ELISAs for detecting exposure to *Brucella ovis*: Molly J. Elderbrook, Brant A. Schumaker, Massaro W. Ueti, Meila Bastos de Almeida, Thallitha S. W. J. Vieira, Rafael F. C. Vieira, Kerry S. Sondgeroth

3. Infection Ecology & Epidemiology 2018, Vol 8. 1500846: Comparisons of brucellosis between human and veterinary medicine: Noah C. Hull and Brant A. Schumaker

4. American Society for Microbiology- Journal of Clinical Microbiology- April 2018, Volume 56 Issue 4 e01894-17: Optimization of *Brucella abortus* Protocols for Downstream Molecular Applications: Noah Hull, Jonathan Miller, David Berry, William Laegreid, Ashley Smith, Callie Klinghagen, Brant Schumaker

5. Journal of Agricultural and Food Chemistry. 2018, 66 ,5707-5712: Detection of Multiple Pathogens in Serum Using Silica-Encapsulated Nanotags in a Surface-Enhanced Raman Scattering-Based Immunoassay: Jing Neng, Yina Li, Ashley J. Driscoll, William C. Wilson, and Patrick A. Johnson

6. **Unit 6723 BRAND - what is the interest rate on the BRAND loans if students do not come back to work in the state?**

*Interest shall begin accruing after the Student's completion of the program but in no event later then 2 years after the student enters into this agreement. Interest shall accrue at an annual rate equal to that charged for federal Stafford loans at the time interest begins to accrue, which rate shall be adjusted annually to match the federal Stafford loan rate. In no event shall the interest rate be greater the 8%.*

The current Stafford rate for July 1, 2021 – June 30, 2022 is 3.73%.

2020-21 a record low rate of 2.75%

## 7. Unit 9601 NCAR list of the projects that UW works with on the super computer

| Allocation Date | PI | Title | Core Hours (millions) | Campaign Storage (TBs) | Data Visualization (hrs) |
|---|---|---|---|---|---|
| July, 2019 | Mavriplis | Computational Study of Wind Turbine Performance And Loading Response to Turbulent Atmospheric In-Flow Conditions | 44.258 | 180 | 5,000 |
| July, 2019 | Liu-Snow | Quantifying snow darkening effect of black carbon in the western US from difference emission sources | 21.400 | 50 | 10,000 |
| July, 2019 | Lu | Synergizing multi-scale models to study the impacts of biomass burning aerosols on Southeast Atlantic stratocumulus | 19.730 | 86 | 8,000 |
| July, 2019 | Liu Dust | Quantifying the impacts of mineral dust on the Arctic climate using NCAR CESMS | 11.870 | 29 | 5,000 |
| July, 2019 | Geerts | Prediction of snow and wind events in the Rocky Mountains (NOAA) | 3.618 | 73 | 10,000 |
| | | | | | |
| December, 2019 | Hannah Jang-Condell | Modeling Planet Formation in Protoplanetary Disks | 1.000 | 0.02 | 5,000 |
| December, 2019 | Liu Xiahong | Radiative impacts of black carbon and brown carbon from wildfires on the Arctic Climate change | 13.200 | 26.50 | 10,000 |
| December, 2019 | Zachary Lebo | Dynamically downscaling CMIP6 GCMs across the western United States to quantify uncertainty in future hydroclimate and fire extremes: WRF experiments conducted on convective-permitting scales* | 23.404 | 293.20 | 10,000 |
| December, 2019 | Zachary Lebo | Analysis of field measurement data to constrain the modeling of winter ozone and its precursors in the Upper Green River Basin | 6.381 | 3.00 | **0** |
| | | | | | |
| July, 2020 | Bart Geerts | Numerical simulations of the impact of cloud seeding in the Wind River Range on precipitation and snowpack | 2.500 | 100.00 | 0 |
| July, 2020 | Jeffrey R. French | Numerical representation of cloud and precipitation growth processes and of the effects of glaciogenic seeding on orographic clouds | 17.200 | 100.00 | 0 |
| July, 2020 | Michael Stoellinger | WRF simulations to generate a Distributed Wind Energy Resource Dataset | 44.200 | 180.00 | 0 |
| July, 2020 | Subhashis Mallick | Advanced reservoir analysis and multitask deep learning - the key tools to enhance fossil-fuel based energy production and address climate change | 1.1 | 10.00 | 0 |
| July, 2020 | Zachary Lebo/Xiaohong Liu | Improving Stratospheric Sulfate Aerosol Representation in GCMs and Investigating Impacts of Volcanic Eruptions on Climate | 9.9 | 0.00 | 0 |
| | | | | | |
| December, 2020 | Bart Geerts | Numerical Simulations of Cold Air Outbreaks Using a Multi-Scale Modeling Framework | 28.340 | 251.00 | 5,500 |
| December, 2020 | Daniel McCoy | Quantifying uncertainty in aerosol-cloud interactions with orbital and suborbital platforms through data-model synergies | 11.300 | 128.80 | 20,000 |
| December, 2020 | Hannah Jang-Condell | Modeling Planet Formation in Protoplanetary Disks | 2.000 | 44.00 | 5,000 |
| December, 2020 | Michael Stoellinger | WRF simulations to generate a Distributed Wind Energy Resource Dataset | 15.7 | 0.00 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| December, 2020 | Zachary Lebo/Xiaohong Liu | Enabling Aerosol-cloud interactions at GLobal convection-permitting scalES (EAGLES) | 31.5 | 172.50 | 10,000 |
| December, 2020 | Zachary Lebo | Quantifying the effects of landscape evolution and mountain uplift in Eastern Asia on the formation of the Chinese Loess Plateau | 6.42 | 157.50 | 100,000 |
| | | | | | |
| July, 2021 | Bart Geerts | Numerical simulations of the impact of cloud seeding in the Wind River Range on precipitation and snowpack | 9.8 | 150.00 | 0 |
| July, 2021 | Subhashis Mallick | A deep learning based approach to address global climate change | 2.75 | 40.00 | 0 |
| July, 2021 | Dimitri Mavriplis | Computational Study of Wind Turbine Performance and Loading Response to Turbulent Atmospheric Inflow Conditions | 26.277 | 106.00 | 5,000 |

## 8. E-mail request from November 24, 2021- WWAMI & WICHE:

**UW Response to LSO Request for Information**
**12/2/21**

**Responses to Inquiries Related to WWAMI**

*Could the UW advise as to:*

*a. Number of available slots;*

**WWAMI: There are currently 20 slots per year, for a total of 80 slots.**

*b. Total number of "funded" slots assuming the standard budget*

**WWAMI: There are 20 WWAMI slots per year, for a total of 80 slots. At the present time, 19 of the 20 slots are fully funded. It is necessary to increase student tuition to fund all 20 slots.**

*c. What would the cost be per additional seat for both programs, e.g., WWAMI would require $X and WICHE, on average, would require $Y.*

**WWAMI: The cost for each WWAMI slot would be approximately $107,038 per year of attendance per student, or $428,151 for the entire 4-year program. We feel that increasing the number of WWAMI slots would be prohibitive for the following reasons:**

a) **Our current slots are not fully funded.**
b) **The WWAMI program receives approximately 65 applicants per year. In any given year, WWAMI is able to attract 20 students who hold exceptional academic credentials. However, the program may not be able to take more than 20 students into the program that meet the admissions criteria on a consistent basis from year-to-year. Consequently, 20 students per class appears to be optimal at the present time.**
c) **It is somewhat difficult to identify an adequate number of physicians around the state who can serve as clinical instructors ("preceptors") to our students in physicians' clinical settings; we want to maintain the high quality of the student experience.**
d) **The number of slots in the WWAMI program has increased gradually over time. Having a class size of 20 is relatively recent, and we have not yet reached the point where the entire first class of 20 students have completed their residencies/fellowships. Until we have 20 WWAMI graduates who are evaluating their prospects for employment in the state of Wyoming on an annual basis, it will be difficult to predict their rate of return to the state and whether increasing future class sizes would be a viable option.**
e) **While there is some room to expand the class size in our current space, we are space-limited and would need to hire additional personnel to accommodate even a modest increase of 4 students into the program.**

In addition to number of seats, is there a need to increase the standard to "fully fund" the existing slots. It appears that special revenue is subsidizing the WWAMI program for this upcoming biennium?

**WWAMI: The WWAMI budget has decreased by $789,974 over the last two fiscal years through a combination of budget cuts to the state appropriation ($574,440) and unfunded increases to our state support contract with the University of Washington ($215,534). It is necessary to address the budget shortfall by increasing the tuition paid by the WWAMI students; this increase is calculated to be $9,875 per student. The most recent submission to increase student payments are reflected in the proposed change to the UW Fee Book, shown below:**

| |
|---|
| **Summary of Proposal:** 4% increase in medical education contract support payments to reflect annual tuition and fee increases at the University of Wyoming School of Medicine, in addition to a $3,328 increase per student due to the decrease in funding from the State of Wyoming. |
| **Proposed Description:**<br>**WWAMI Medical Education***<br>**1. Year 1 contract support payment: $21,171**<br>**2. Year 2 contract support payment: $22,018**<br>**3. Year 3 contract support payment: $22,899**<br>**4. Year 4 contract support payment: $23,815**<br>***Please note these rates are for students entering into contracts in FY 23. All previous rates are outlines in the individual contracts** |

**In order for the program to be funded at level that was originally intended, we need to increase state funding to $6,529,409 to account for these increases to the contract with UWSOM, and take steps to ensure that budget cuts do not affect the WWAMI program and future increases to the contract are covered.**

**During the 2021 budget session, a footnote directed the UW to explore a different affiliation for medical education. Multiple conversations were had with the leadership of Colorado University School of Medicine (CUSOM), and a proposal to affiliate was explored with the Dean of the CUSOM. The CUSOM Dean intimated it was not a good time to explore a new affiliation, as the COVID-19 pandemic has dramatically increased the caseloads in their facilities above capacity and has reduced their ability to take more students at this time. Further, with what appears to be the imminent opening of a School of Osteopathy in Greeley, the CUSOM will most likely be faced with a decrease in clinical rotation sites for its current students. Consequently, the CUSOM is not able to commit to having UW students at the Denver tertiary referral hospital locations, which is a requirement for quality medical education.**

**We would welcome a discussion regarding the priorities of the legislature as it pertains to a potential new affiliation so that we can best meet this request.**

## Responses to Inquiries Related to WICHE

*Could the UW advise as to:*

**a.** *Number of available slots;*

**WICHE: The number of slots available is typically discipline-specific (see below), and can be changed from year-to-year to meet current demands. WICHE Professional Student Exchange Program (PSEP) does not fund a specific number of slots. Rather, the currently funded students are subtracted from the budget and then potential slots are divided up between the 8 professional fields funded by PSEP. Number of students accepted into PSEP programs and who also are willing to sign the contract*, is highly variable from year to year, so funds are inevitably reallocated between fields. Costs of "slots" range from $14,300 for Occupational Therapy to $34,175 for Allopathic Medicine, with a financial commitment between 2 to 4 years depending upon field.**

**While we could use previous years to calculate an average cost of a "slot" it would be unlikely to realistically reflect the costs of future year's students.**

**For the last couple of bienniums, the WICHE PSEP budget has ended in the black. This seems to be due to students not being willing to sign the contract which commits them to returning to Wyoming. The reduction of seats in the WYDENT Program may result in an increased number of dental students using WICHE PSEP and absorb this excess--but that remains to be seen.**

*b. Total number of "funded" slots assuming the standard budget*

**WICHE: The slots committed for FY22 (new and ongoing slots) are as follows:**

| Discipline | Total Slots | Total Cost |
|---|---|---|
| Dental School | 2 | $ 56,000 |
| Medical School | 11 | $ 367,400 |
| Occupational Therapy | 5 | $ 102,697 |
| Optometry | 7 | $ 134,925 |
| School of Osteopathy | 4 | $ 95,800 |
| Physical Therapy | 21 | $ 338,625 |
| Physician Assistant | 1 | $ 19,075 |
| Podiatry | 0 | $ - |
| Veterinary Medicine | 19 | $ 928,200 |

*c. What would the cost be per additional seat for both programs, e.g., WWAMI would require $X and WICHE, on average, would require $Y.*

**WICHE: The cost per slot in the WICHE Program is discipline-specific; it ranges from $14,300 for Occupational Therapy to $34,175 for Allopathic Medicine, with a financial commitment between 2 to 4 years depending upon field.**

**While we could use previous years to calculate an average cost of a "slot" it would be unlikely to realistically reflect the costs of future year's students.**

In addition to number of seats, is there a need to increase the standard to "fully fund" the existing slots.

**WICHE: In general, the number of slots across disciplines seems to be meeting the demand. Consequently, barring annual increases in WICHE costs, the current funding is adequate. However, if the demand for certain disciplines (such as Physical Therapy) should increase, then current funding will not be adequate unless slots in other disciplines are reduced.**

9. **E-mail request from January 2, 2022:**
    a. **Please clarify whether Wyoming's computing time allotment is subject to NSF (or other federal entity, UCAR, NCAR, etc.) oversight and to what level or whether the competition of computing time for UW's allotment is subject to internal review?**

The partnership between UCAR and UW regarding the NWSC is based on an MOU, and overseen by the Project Governance Board (PGP).

The Board is typically comprised of the UCAR President, the NCAR Director, the UW President, and the UW Provost or VP for Research and Economic Development. The allocations are overseen by the Wyoming Resource Allocation Panel (WRAP) which is co-chaired by a scientist at NCAR and a faculty member at UW (currently Professor Suresh Mukhnallipatna of the Electrical and Computer Engineering department). The WRAP reviews all large allocation requests for the Wyoming share of the NWSC and makes recommendations to the PGB. The WRAP co-chairs review all small allocation requests.

The policies for the Wyoming allocations were developed when the NWSC was in its planning stages, and were subject to NSF approval.

Details about the WRAP and the policies can be found at http://www.uwyo.edu/nwsc/. Particular items to note:

a) all Wyoming allocations require a UW or CC faculty or student as the lead,
b) the scientific scope of the projects eligible for the Wyoming Allocation is much broader than for typical NCAR allocations (which are restricted primarily to Atmospheric Sciences) and include anything related to the broadly defined areas of earth system science (see http://www.uwyo.edu/nwsc/Eligibility/science_areas.html).
c) The primary factors limiting Wyoming researchers from use of the NWSC are the architecture (the NWSC systems is not optimal for applications), and the requirement that the resources cannot be used for commercial purposes.

The WRAP is currently reviewing projects for December 2021/January 2022 allocations.

# APPENDIX A

*Ecological Applications*, 30(6), 2020, e02129


# Chronic wasting disease undermines efforts to control the spread of brucellosis in the Greater Yellowstone Ecosystem

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*Abstract.* Wildlife diseases pose a substantial threat to the provisioning of ecosystem services. We use a novel modeling approach to study the potential loss of these services through the imminent introduction of chronic wasting disease (CWD) to elk populations in the Greater Yellowstone Ecosystem (GYE). A specific concern is that concentrating elk at feedgrounds may exacerbate the spread of CWD, whereas eliminating feedgrounds may increase the number of elk on private ranchlands and the transmission of a second disease, brucellosis, from elk to cattle. To evaluate the consequences of management strategies given the threat of two concurrent wildlife diseases, we develop a spatiotemporal bioeconomic model. GPS data from elk and landscape attributes are used to predict migratory behavior and population densities with and without supplementary feeding. We use a 4,800 km$^2$ area around Pinedale, Wyoming containing four existing feedgrounds as a case study. For this area, we simulate welfare estimates under a variety of management strategies. Our results indicate that continuing to feed elk could result in substantial welfare losses for the case-study region. Therefore, to maximize the present value of economic net benefits generated by the local elk population upon CWD's arrival in the region, wildlife managers may wish to consider discontinuing elk feedgrounds while simultaneously developing new methods to mitigate the financial impact to ranchers of possible brucellosis transmission to livestock. More generally, our methods can be used to weigh the costs and benefits of human-wildlife interactions in the presence of multiple disease risks.

*Key words:* *brucellosis; chronic wasting disease; cost–benefit analysis; elk feedgrounds; Greater Yellowstone Ecosystem; spatiotemporal models.*

## Introduction

The impending introduction of chronic wasting disease (CWD) to the Greater Yellowstone Ecosystem (GYE) is threatening one of our iconic ecosystems. The 100-yr-old practice of supplemental feeding of GYE elk, which has successfully limited the spread of brucellosis from elk to livestock by limiting elk movement onto ranches, may exacerbate the spread of CWD in the elk population by enhancing opportunities for CWD to spread among elk (National Academies of Sciences, Engineering, and Medicine 2017). To investigate how CWD will impact the provisioning of ecosystem services within the GYE and how the many distinct elk feedgrounds affect the risks to these services, it is critical to understand how disease transmission varies over the spatial landscape. Incorporating a spatial dimension into models of coupled ecological–economic systems allows for a richer understanding of the tradeoffs and synergies associated with ecosystem service provisioning and optimal management (Bulte et al. 2004, Qiu and Turner 2013).

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Bioeconomic models of coupled human–natural systems have been developed to study the management of wildlife disease and have been recommended as tools for managing disease in the GYE (National Academies of Sciences, Engineering, and Medicine 2017). The majority of bioeconomic models of disease management are aspatial and thus may be limited in applications where the economic and ecological impacts of management strategies may be spatially heterogeneous. To address this limitation, we develop a spatially explicit bioeconomic model of the GYE to examine the management of two infectious diseases carried contemporaneously by elk: CWD and brucellosis. A spatially explicit model is advantageous in that it can generate: (1) a more accurate assessment of economic risks (i.e., the combination of adverse ecological outcomes arising within the coupled spatial system, and the associated economic consequences arising across a heterogeneous landscape; Perrings 2005) and of how various interventions can mitigate these risks, and (2) improved species and disease management recommendations that may be spatially explicit to target areas where strategies can generate the largest net benefits.

Supplementary feeding of elk in the southern GYE during the winter and spring has been in effect since the early twentieth century to reduce winter mortality and support larger elk herds than could be sustained by natural forage alone. The larger elk herds in turn provide significant economic benefits to hunters and those who value wildlife viewing (Smith 2001, National Academies of Sciences, Engineering, and Medicine 2017). Additionally, feedgrounds help truncate the natural migratory routes of elk, thereby limiting the time elk spend on private, low-elevation ranchlands during the winter months and reducing the risk of brucellosis transmission from elk to cattle. Brucellosis risk to cattle is currently the primary GYE disease concern, and ranchers incur large regulatory costs to prevent brucellosis from spreading beyond the Designated Surveillance Area (DSA) (National Academies of Sciences, Engineering, and Medicine 2017). If a cow becomes infected, the U.S. Department of Agriculture–Animal and Plant Health Inspection Service (USDA-APHIS) requires the entire cattle herd and all contact herds to be quarantined or culled, at a significant cost to either the rancher, the states affected, the U.S. Department of Agriculture (USDA), or all three (Roberts et al. 2012).

Supplemental feeding is increasingly challenged by wildlife biologists, ecologists, and epidemiologists (Smith 2012, National Academies of Sciences, Engineering, and Medicine 2017). A major concern is the practice increases brucellosis prevalence in elk by concentrating elk populations at feedground sites (Schumaker 2010, Scurlock and Edwards 2010, Smith 2012). Feedground opponents argue that the long-term costs of increased prevalence exceed the disease protection benefits of feedgrounds. This concern has grown as CWD has spread across Wyoming and will likely soon be introduced into GYE elk populations (see Appendix S1).

The economic costs of a CWD outbreak in and around the GYE elk feedgrounds have not previously been estimated, but would negatively affect two key sectors of the region's economy: tourism and hunting. First, over four million tourists visit Yellowstone National Park every year and almost nine million visit the state of Wyoming, with many coming to view elk and other wildlife. CWD is an infectious neurodegenerative wildlife disease that causes certain death for its hosts, with infected animals being noticeably sick during latter stages of disease. Second, the public remains wary of consuming meat of infected animals, even though there is very little evidence of transmission to humans (Belay et al. 2004). Indeed, hunting activity and expenditures in Wisconsin declined following the 2002 discovery of CWD in deer populations, resulting in economic losses of between $53 and $79 million in 2002 and between $45 and $72 million in 2003 (Bishop 2004). Zimmer et al. (2012) find hunters in Alberta would be willing to spend $20.35 per trip to prevent the incidence of CWD from increasing beyond current levels.

An important emerging consideration for GYE elk management, particularly in the face of disease transmission, is how elk move and congregate in space (Merkle et al. 2018). Brucellosis transmission (and likely future CWD transmission) among elk, and also to local cattle, depends on how spatially explicit management, such as feedgrounds, affect population densities and elk movement throughout the year. Previous bioeconomic models of wildlife disease management involving supplemental feeding (Horan and Wolf 2005, Fenichel and Horan 2007b) have been aspatial. Aspatial models must make strong assumptions about how changes in feeding affect animal densities and resultant disease transmission, both within the elk population and to cattle, which may greatly oversimplify calculations of transmission likelihoods and ensuing economic impacts. As indicated above, the spatial impacts of feedgrounds on elk migration patterns affecting cattle risks are considered especially important (Jones et al. 2014).

Spatial models have been critical for understanding and designing strategies for addressing a variety of environmental issues such as climate change, pollution dynamics, wildlife migration, and land use (Veldkamp and Lambin 2001, Pearson and Dawson 2003, Guisan and Thuiller 2005, Jerrett et al. 2005). For example, spatial pollution models are more accurate for assessing health and ecosystem impacts, and for designing spatially explicit policies that can mitigate these impacts more cost effectively. Spatially explicit modeling of elk movement helps advance our understanding of how alterations in supplemental feeding can be used to influence elk densities across space and wildlife disease transmission. A spatial model is also important because CWD can be passed to elk through environmental contamination, so it is important to keep track of where elk

currently reside and where they have been in the past. Aspatial models are often used to assess economically efficient management because of the difficulty of integrating human behavior with biological systems. We address this difficulty by coupling realistic and practical elk management strategies with a spatial bioeconomic model to assess the welfare (discounted flow of ecosystem net benefits) associated with current supplemental feeding policies and counterfactual policies where supplemental feeding is either eliminated or reduced in a spatially strategic manner. Our hypothesis is that the introduction of CWD will alter the costs and benefits of supplemental elk feeding and require new elk management strategies that will redistribute the costs and benefits to stakeholders in the region.

Results indicate that with the introduction of CWD into these elk populations, the additional risk feedgrounds generate outweighs the benefits they provide. With the introduction of CWD and our proposed adapted management practices, the distribution of ecosystem services changes and leads to a situation where certain stakeholders may require compensation for their diminished level of ecosystem services.

## Models and Methods

Our study area is a 4,800-km$^2$ area around Pinedale, Wyoming, USA, which contains four existing feedgrounds. This area is at the southwestern slope of the Wind River Mountain Range within Sublette County and one of the southernmost portions of the GYE. We chose this area for two reasons: (1) elk in this area are likely to be some of the first in the GYE to encounter CWD and (2) wolves are not present in large numbers. In areas farther north, wolves play an important role in elk population dynamics, and it is also hypothesized that predation may play a role in regulating disease in prey populations (Wild et al. 2011). The study area is broken down into a 12 × 16 grid of 25-km$^2$ cells (Fig. 1). Simulations are used to generate welfare estimates under a variety of harvesting and feeding management strategies.

The model contains one wildlife species (elk), two diseases (CWD and brucellosis), and one livestock species (cattle). We treat brucellosis as endemic in the elk population whereas CWD is modeled as being newly introduced to the study area. The total elk population, which consists of subpopulations defined by health status (e.g., susceptible, infected), is denoted $N_{i,t}$, where $i \in \{1, \ldots n\}$ indexes distinct patches of land or cells and $t \in \{1, \ldots, T\}$ indexes time. Monthly time steps are used to capture the seasonal migratory behavior of elk and how this behavior is affected by feeding. To model elk population changes, we establish an order of the population-related events or stages that may (but do not necessarily) occur within a month. The first stage is elk population growth. The second stage is elk hunting. The third stage is elk mortality from CWD, assuming that CWD is always terminal (Williams et al. 2002). The fourth stage is disease infection dynamics, which includes elk-to-elk, elk-to-environment, and environment-to-elk disease transmission as well as the transmission of brucellosis to livestock. The fifth stage is animal movement.

### *Stages 1–3: Elk growth, hunting, and CWD mortality*

Elk growth, recruitment less natural mortality, is assumed to occur only at the beginning of June; stage 1 does not occur in any other month. For each cell, the elk population exhibits logistic growth, with an intrinsic growth rate denoted $r$ and carrying capacity, $K$. Carrying capacity, $K(F)$, is modified to be an increasing function of the quantity of supplemental feeding, $F$ (Walters 2001). Unlike the spatial model in Horan et al. (2005), carrying capacity applies to the entire region rather than each cell because the case study area is relatively small.

The regional planner (e.g., Wyoming Game and Fish Department) determines the total number of elk to be harvested in each October, $h_t$; no harvests occur in other months. For simplicity and because the Pinedale region is a comparatively small region in the GYE, elk harvests are specified for the entire region, with harvests on both public and private lands being distributed proportionately to the total Pinedale elk population (this latter assumption is relaxed in our sensitivity analysis in Appendix S1). Moreover, elk hunting is distributed proportionally across the infected and susceptible populations because selective harvesting is difficult, except in the later stages of the disease. In each period, the CWD-infected elk population is reduced from CWD mortality according to a fixed CWD mortality rate, μ.

### *Stage 4: Disease transmission*

*CWD transmission.*—Chronic wasting disease elk-to-elk dynamics are modeled using an SI compartmental model where $S_{i,t}$ and $I_{i,t}$ are the number of susceptible (CWD-free) and CWD-infected elk in cell $i$ at time $t$, with $N_{i,t} = S_{i,t} + I_{i,t}$ (recall that CWD is always fatal). For future reference, we also denote $\theta_{\text{CWD},i,t} = I_{i,t}/N_{i,t}$ as the prevalence of CWD in elk. The number of new CWD infections in cell $i$ at time $t$ is modeled according to the standard density-dependent transmission function $\beta_{\text{CWD}} S_{i,t} I_{i,t}$ (McCallum et al. 2001, Begon et al. 2002), where $\beta_{\text{CWD}}$ is the infection coefficient. Transmission may vary considerably across cells due to differences in cell-specific population densities. There is considerable uncertainty regarding CWD transmission rates. Given this uncertainty, we carefully explain our calibration procedure and we also perform a sensitivity analysis in Appendix S1. Note that the calibration is scale-dependent so that if we had increased the resolution of the model (i.e., smaller cells), the CWD transmission parameter would adjust so as to have little impact on equilibrium disease prevalence and transmission rates. We also note that other transmission functions are possible

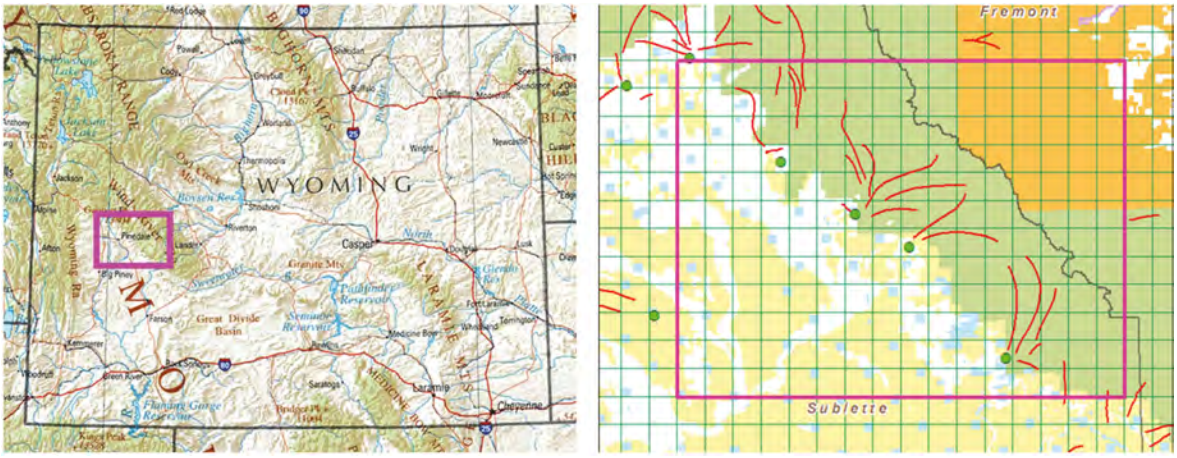


FIG. 1. Case study area with four elk feedgrounds near Pinedale, Wyoming, USA. The green dots on the right graph indicate the location of the four elk feedgrounds. The red lines indicate approximate elk migration routes provided by the Wyoming Game and Fish Department. Green indicates U.S. Forest Service land, yellow indicates U.S. Bureau of Land Management land, and white indicates private land.

(McCallum et al. 2001) and are investigated in Appendix S1.

New CWD infections from environment-to-elk transmission are given by $\beta_{PRION} S_{i,t} P_{i,t}$, where $\beta_{PRION}$ is the environment transmission parameter and $P_{i,t}$ is an environmental contamination state variable that varies across space and time. Variable $P_{i,t}$ indicates the level of prion contamination in the environment due to CWD-infected elk residency and mortality. The law of motion for this state variable is

$$P_{i,t+1} = (1 - \gamma_{PRION}) P_{i,t} + \delta_{PRION} I_{i,t} \quad (1)$$

where $\gamma_{PRION} > 0$ is the slow decay rate (Saunders et al. 2008) and $\delta_{PRION}$ is the elk-to-environment transmission parameter.

Transmission may vary considerably across cells due to cell-specific population densities and environmental conditions. Aspatial models that include feeding generally model $\beta$ as a function that is increasing in feeding (Horan and Wolf 2005, Fenichel et al. 2010). An advantage of our spatial modeling approach is that the transmission function does not need to be modified based on feeding decisions. This is because population densities, and hence transmission, in a cell respond to relative feeding opportunities in that cell.

*Brucellosis.*—Unlike CWD, brucellosis is already endemic in GYE elk populations and spreads occasionally from elk to cattle. Transmission of brucellosis from elk to cattle is modeled as density dependent, with the probability of a cow being newly infected with brucellosis in cell $i$ at time $t$ given by $\beta_{BRUC} \theta_{BRUC,t} N_{i,t}$, where $\beta_{BRUC}$ is the brucellosis transmission parameter. Here, $\theta_{BRUC,t}$ is the prevalence of brucellosis in elk and $N_{i,t}$ is the total number of elk in the cell, so that the number of infected elk in cell $i$ at time $t$ is $\theta_{BRUC,t} N_{i,t}$. An SIR model is not used for elk brucellosis dynamics. Rather, we assume $\theta_{BRUC,t}$ transitions to one of two steady-state prevalence levels depending on whether the elk population is fed or unfed. Scurlock and Edwards (2010) estimate a prevalence of 3.7% in unfed populations and 21.9% in fed populations. Schumaker (2010) reports rates of less than 5% in unfed populations and 26% in fed populations. Recent data in unfed elk herds in the GYE show evidence of increasing brucellosis prevalence in unfed elk populations (see Appendix S1 for further details). Based on this evidence, we initially assume that brucellosis is 26% in both unfed and fed elk populations. In the *Sensitivity Analysis* section of Appendix S1, we allow prevalence levels to be different and transition between the two prevalence levels. A convergence parameter, $\delta_{BRUC}$, governs the rate of this transition.

Feeding affects brucellosis transmission to cattle in two ways. First, the larger prevalence level due to feeding means elk that come into contact with cattle are more likely to be infected, increasing risks to cattle. Second, feeding reduces the number of elk that travel into lower elevations and inhabit the same space as cattle. Because the primary mechanism of elk-cattle brucellosis transmission is cattle coming into contact with aborted elk fetuses (abortions are the result of brucellosis infections), we assume that transmission to cattle only occurs between January and June.

### *Stage 5: Elk movement*

We assume there is no difference in the movement of infected and susceptible elk. The likelihood of an elk moving from any cell $i$ to any cell $j$ in stage 5 is governed by an $n \times n$ transition matrix $J$. $J$ is calculated by taking the Hadamard product (element by element

multiplication) of two $n \times n$ matrices and then normalizing the columns to sum to 1. The first matrix is a movement matrix, $M$. Each element of $M$ gives the probability of an elk moving the distance required to reach a point in cell $j$ from the center of cell $i$ in a month, if following "rook" movement (i.e., elk move due north, south, east, or west. See Appendix S1: Figs. S1, S2 and Section S1.5). The elements of the second matrix, $Z_{m,F}$, are probabilities of an elk inhabiting a particular cell divided by the probability of an elk inhabiting some other location within its home range (an odds ratio), conditional on the landscape characteristics of the cell. These values are generated by fitting a resource selection function (RSF) to data on elk movement and GYE habitat characteristics, such as elevation and available green plant biomass. There are 24 different $\mathbf{Z}$ matrices, one for each month for each type of elk population (fed and unfed). These methods are based on many of the principles of the Master Equation approach to calculating animal space use outlined in Merkle et al. (2017). A detailed description of the movement methodology, along with the RSF estimation procedure and parameter estimates, can be found in Appendix S1: Sections S1.5, S2.5. Noting that each column of $J$ sums to 1 so that every elk has to either stay in place or travel to another cell, the movement of elk in stage 5 is given by

$$S_{t+1} = JS_t \tag{2}$$

$$I_{t+1} = JI_t \tag{3}$$

where $S$ and $I$ are $n \times 1$ column vectors of the susceptible and infected populations.

Fig. 2 shows heat maps of predicted elk population densities in March simulated by our movement model under two cases. In the case where all feedgrounds are open, elk are all concentrated around the feedground sites. In the case where all feedgrounds are closed, elk are less concentrated but more are located on low elevation, private land. In August, however, the two heat maps look similar (see Appendix S1: Figs. S3, S4).

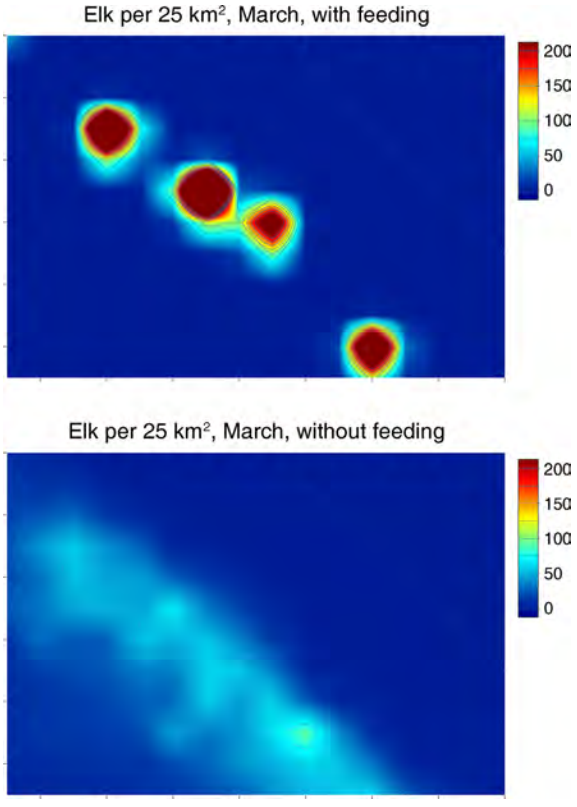


FIG. 2. Elk population densities with and without feedgrounds for the case study area. The top graph shows the prediction of our movement model for the status quo scenario in which elk are fed during the winter. All elk are concentrated around feedground locations with a few on private land. The bottom graph shows the prediction of our movement model for the counterfactual scenario in which elk feedgrounds are closed. Elk are less concentrated but are more prevalent on private land. An interpolation method is used to smooth the population densities.

### *The bioeconomic model*

A bioeconomic model is used to track the economic and ecological incentives for optimal management. Regional welfare consists of net hunting benefits less brucellosis and biosecurity costs incurred by ranchers. We assume CWD only affects hunters' welfare with two negative economic consequences. First, mortality from the disease reduces the elk population size from which to harvest, increasing harvest costs and therefore reducing hunter demand (Kauffman et al. 2012). Second, the presence of CWD in a region causes a shift in demand as hunters may choose to hunt elsewhere to reduce their risk of harvesting an infected animal (Bishop 2004, Zimmer et al. 2012). To capture this demand shift, we model net marginal willingness to pay (net of hunting expenditures) as a decreasing function of CWD prevalence. For simplicity and because the Pinedale region contributes only a small portion of regional elk harvests, we assume the marginal value of CWD-free elk harvests in this region is fixed and that the region acts as a price-taker with respect to quantity of licenses issued.

Let $\underline{Y}$ be the constant net marginal value of hunting CWD-free elk in the Pinedale region. Following Schumaker (2010), the net marginal value of harvesting CWD-infected elk is zero. This means the aggregate net marginal value of hunting in the Pinedale region in time $t$ is

$$Y_t = \left(1 - \theta_{\mathrm{CWD},t}\right)\underline{Y} \tag{4}$$

where $\theta_{\mathrm{CWD},t}$ is regional CWD prevalence. Total regional hunting welfare in period $t$ is $h_t Y_t$. The sensitivity analysis in Appendix S1 includes scenarios with lower hunter demand response to CWD.

A potential limitation is that the prescribed reductions in elk populations may not be feasible in the short term through hunting only, particularly if hunters do not have access to private lands. If elk depopulation to achieve a population target also required efforts from the Wyoming Game & Fish Department (WGFD), then these additional harvests would create an agency cost rather than a hunting benefit. This was confirmed through personal communication with WGFD officials. To account for this possibility, we assume there is a maximum number of elk that can be successfully hunted within the year and that the WGFD organizes any additional harvests at a fixed cost per elk. Harvest welfare is therefore the difference between hunting benefits and (if necessary) agency depopulation costs. See Appendix S1 for further details regarding agency depopulation costs.

It is assumed that ranchers are running cow–calf operations, which are the primary type of operation in the GYE and the type primarily at risk from brucellosis. These herds are quarantined if a brucellosis infection is detected, a regulatory response that has become more common than whole-herd depopulation given USDA and state budget limitations, in addition to rule changes (Roberts et al. 2012). We denote the per-cow cost of a quarantine $q$. For simplicity, assume that the $L_{i,t}$ cows in any individual cell at time $t$ constitute a herd. The herd has to be quarantined for at least one year if one or more cows contract the disease from elk. Quarantine costs (damages to ranchers) in a cell are independent of the number of cattle brucellosis infections; after the first infected cow is detected and the herd is quarantined, there are no additional costs if more cows in the herd become infected in the same period. Recognizing that the expected number of brucellosis infections in each cell follows a binomial distribution, the probability of at least one cow becoming infected is

$$1 - (1 - \beta_{\text{BRUC}}\theta_{\text{BRUC},t}N_{i,t})^{L_{i,t}}. \quad (5)$$

Assuming perfect disease monitoring, Eq. 5 can be interpreted as the probability of a quarantine in cell $i$ at time $t$. In the absence of any measures to reduce the risk of cattle contracting the disease, the expected economic damages that ranchers in the Pinedale region incur from brucellosis at time $t$, absent any self-protection measures (described below), are

$$D_t = q\sum_{i=1}^{n} L_{i,t}\left(1 - \left(1 - \beta_{\text{BRUC}}\theta_{\text{BRUC},t}N_{i,t}\right)^{L_{i,t}}\right). \quad (6)$$

In addition to damages from brucellosis, we also include elk-dependent costs such as destruction of fences, damages to crops, and general forage depredation from elk on private land. These depredation costs are assumed to be proportional to the number of elk such that the total costs on private land are given by $c_N \times N_{i,t}$, where $c_N$ is the monthly cost per elk. Each cell is either denoted as private or public land, but only cells denoted as private land are subject to depredation costs.

Ranchers can invest in self-protection measures to reduce the risk of brucellosis infection during the winter. These measures have varying levels of cost, ranging from low-cost options such as vaccination to high-cost options such as building elk-proof fence and delaying grazing (Roberts et al. 2012). For simplicity, we assume ranchers can choose a level of self-protection against brucellosis infection as represented by the indicator variable, $\Phi_{i,t} \in [0, 1]$, where $\Phi_{i,t} = 0$ indicates no protection and $\Phi_{i,t} = 1$ indicates full protection. The effectiveness of the self-protection is given by the function $\varphi_{i,t} = \varphi_0\Phi_{i,t}^{\varphi_1}$ such that the probability of at least one cow in a cell becoming infected in expression (5) is reduced by $100 \times \varphi_{i,t}$ percent. The per-cow total cost of self-protection is given by the function $c_{i,t} = c_0\Phi_{i,t}^{c_1}$, which is reduced for the rancher by $100 \times c_{\text{GOVT}}$ percent through government subsidies.

For the herd located in cell $i$ at time $t$, with $L_{i,t}$ cows, a risk-neutral rancher will invest in self-protection up to the point where the expected marginal reduction in damages equals the marginal cost of self-protection, i.e.

$$\varphi_{i,t}' q L_{i,t}\left(1 - \left(1 - \beta_{\text{BRUC}}\theta_{\text{BRUC},t}N_{i,t}\right)^{L_{i,t}}\right) = c_{i,t}' L_{i,t}(1 - c_{\text{GOVT}}) \quad (7)$$

where $\varphi_{i,t}'$ and $c_{i,t}'$ are the derivatives of the effectiveness and cost functions with respect to $\Phi_{i,t}$, respectively. If the expected marginal reduction in damages are always greater (less) than the marginal private cost of self-protection, then the optimal level of self-protection is $\Phi_{i,t}^* = 1$ ($\Phi_{i,t}^* = 0$). With self-protection, the expected economic damages from brucellosis in period $t$ become

$$D_t = q\sum_{i=1}^{n} L_{i,t}\left(1 - \varphi_{i,t}^*\right)\left(1 - \left(1 - \beta_{\text{BRUC}}\theta_{\text{BRUC},t}N_{i,t}\right)^{L_{i,t}}\right) \quad (8)$$

where $\varphi_{i,t}^*$ is the effectiveness of optimal self-protection $\Phi_{i,t}^*$. The total self-protection costs include those incurred by both ranchers and the government. With $c_{i,t}^*$ denoting the self-protection costs that are optimal to ranchers, total costs are

$$\sum_{i=1}^{n} c_{i,t}^* L_{i,t}. \quad (9)$$

### *Welfare function*

A regional planner concerned with societal economic efficiency seeks to maximize the discounted sum of expected economic welfare, $W$, by choosing the elk harvest levels, $h_t$, and by deciding whether or not to provide supplemental feed to elk, subject to rancher self-

protection choices in response to brucellosis risks. For simplicity, we specify $F_{i,t}$ as a binary variable such that $F_{i,t} = 1$ indicates feeding at current levels and $F_{i,t} = 0$ indicates no feeding. Some simulations will involve cell-specific feeding while others will involve a single feeding choice for the region. Discounted expected welfare is

$$W = \sum_{t=0}^{T} \frac{1}{(1+\rho)^t} \begin{pmatrix} h_t Y_t - D_t - \sum_{i=1}^{n} c_N N_{i,t} \\ - \sum_{i=1}^{n} c^*_{i,t} L_{i,t} - z \sum_{i=1}^{n} N_{i,t} F_{i,t} \end{pmatrix} \quad (10)$$

where $z$ is the cost of feed per elk and $\rho$ is the discount rate. Discounting over time is standard in the economics literature and implies that earlier time periods will receive a larger weight in the welfare function. This is because individuals generally prefer receiving benefits now rather than later (Arrow et al 1996). As a reminder, the components of welfare in Eq. 10 from left to right are (1) hunting benefits, (2) expected brucellosis damages, (3) elk depredation costs on private land, (4) optimal brucellosis self-protection costs, and (5) supplemental feeding costs.

### *Elk management practices*

We focus on three elk management practices that are relatively transparent and straightforward to implement: fixed population target (FPT), fixed harvesting rate (FHR), and population target switching (PTS). The two "fixed" alternatives involve fixing either the target population size or the hunting rate as a percentage of the elk population and restricting them to be the same in every period. The "switching" alternative is analogous except it allows the flexibility to alter the population target if the prevalence of CWD is sufficiently low. Each of these scenarios model brucellosis and CWD risks, with one exception: the FPT and current management practices are also modeled for the case where there are only brucellosis risks (no CWD). These no-CWD scenarios are considered the baseline scenarios, as most existing discussions of disease management ignore the effects of CWD (Bienen and Tabor 2006) and focus on the management of brucellosis transmission risks to livestock. Each strategy is selected given the self-protection measures chosen by ranchers.

We also evaluate two types of feeding strategies. First, each alternative indicated above is evaluated under two feeding options that are not spatially differentiated: feed at the (constant) status quo levels or to discontinue feeding at all feedgrounds. Second, we consider spatial management strategies under the FPT practice with CWD where all possible subsets of feedgrounds are closed. There are four feedgrounds and 16 possible configurations where anywhere from zero to four feedgrounds are closed. Since we already consider the cases where no feedgrounds are closed and all feedgrounds are closed, there are an additional 14 spatial configurations to evaluate.

A search algorithm is used to identify the population target (and mix of open and closed feedgrounds in the spatial feedground case) that produces the most economically efficient outcome, i.e., that maximizes the present value of net economic benefits as given by Eq. 10, given the available management tools examined here. For the FPT practice, the number of elk hunted each year is determined by taking the difference between the current population and a population target that remains constant over time. With an FHR practice, a fixed percentage of elk are hunted each year. The PTS practice determines a number of elk hunted each year by taking the difference between the current population and one of two population targets: one target is used if the CWD prevalence is below a population management threshold, $\varepsilon$, and another is used if the CWD prevalence is above this threshold. The threshold is exogenous and meant to represent the level when CWD prevalence is sufficiently low in the relevant elk population. There is not a similar threshold for brucellosis since brucellosis dynamics are not modeled apart from prevalence transitioning in response to changes in the feeding regime.

Simulations start at the beginning of March. At this time of year, almost all elk are located around feedgrounds. The number of elk started at each feedground corresponds to the latest available population counts. The names and latest publicly available population estimates for the four feedgrounds are Soda Lake (1,017), Scab Creek (668), Muddy Creek (571), and Fall Creek (648) (see Appendix S1 for further details). Brucellosis prevalence is initially assumed at the steady state value for a feeding regime, 26%, consistent with the current practice of feeding elk. CWD is introduced exogenously to the Scab Creek Feedground; this introduction is likely to occur by infected deer herds coming into contact with the elk population. It is assumed that 87 elk are initially infected with CWD, which corresponds to an initial prevalence level of approximately 3% for the entire initial study area population of 2,904. Simulations are run for 100 yrs, but discounting causes the first couple of decades to have a significantly higher weight in determining the strategy that maximizes social welfare.

## Simulation Results

The simulation results for the various scenarios are presented in Table 1, with ecological and economic tradeoffs depicted in Fig. 3. We reiterate that a spatially explicit model is a key component in developing an efficient management strategy. CWD transmission depends on where elk are currently located, and environmental transmission depends on where elk have resided in the past. Brucellosis transmission from elk to cattle, although a fairly rare occurrence, also depends on the location of elk. A spatially explicit model is required to accurately measure these dual disease risks.

First consider the current management practice scenario (without CWD). Here we see that social welfare is driven by harvest welfare, with comparatively small agricultural and feeding costs (92% and 68% smaller than harvest welfare, respectively). This result, which is in contrast to traditional GYE concerns about disease impacts to agriculture, arises here because cattle quarantines, while costly, are rare events. The relatively significant role of harvest welfare also drives the optimal strategies in the alternative scenarios we consider. We now turn to these other scenarios.

### *Fixed population target*

First consider the FPT strategy with no CWD. When feeding occurs, the population target is increased 55% and social welfare is increased by 11% relative to the current strategy. Agricultural costs increase by 44% and feeding costs increase by 51% in this scenario. However, because these costs were comparatively small to begin with, the impacts on social welfare are determined primarily by the 26% ($15.75 million) increase in the present value of harvest net benefits. Now consider the case where feeding is discontinued. We have calibrated the model such that social welfare is unchanged in this particular scenario (see Appendix S1 for more details about this calibration, and the sensitivity analysis where parameters and the assumptions about hunter access to private lands and CWD transmission functions are varied). In this regard, our analysis is neutral on the question of whether supplemental feeding is economically optimal under the FPT strategy prior to the introduction of CWD. Still, this scenario provides insight into how discontinuing feeding alters the optimal population target and the consequent allocation of costs and benefits to hunters and farmers. The population target is reduced 31% when feeding is discontinued, primarily because harvest opportunities are diminished by reduced ecological productivity (Fig. 3). Note that agricultural costs increase very little without supplemental feeding due to the smaller population target and ability to self-protect against brucellosis infection risk.

Now consider the FPT strategy with CWD. Relative to the case of no CWD, the population target is reduced 79% and social welfare is reduced 68% when CWD risks are present and feeding occurs. This is largely because CWD significantly reduces elk productivity even at moderate population target levels (Fig. 3), resulting in significant adverse welfare impacts to hunters. Some of these adverse impacts are offset by choosing a much smaller target in the presence of CWD risks. Herein lies an important trade-off: all else being equal, the smaller target means increased harvest costs and less ecological productivity to support harvesting activities, but a larger target would fuel CWD transmission to produce a larger decline in ecological productivity so that even fewer harvests would be sustainable. As in the current strategy scenario, the welfare reduction in the case of CWD risks are primarily due to reduced harvest welfare (72%), stemming from a much smaller elk population. Agricultural costs (i.e., expected brucellosis quarantining, brucellosis self-protection and depredation) decline by 74%, but these costs are relatively small in comparison to the other economic impacts and therefore have less of an impact on social welfare.

Chronic wasting disease has a smaller impact on population targets (31% reduction relative to FPT with no feeding or CWD) and welfare (20% reduction) when

Table 1. Summary of simulation results under various management practices and feeding scenarios in the case study area.

| Management practice | Feed | Elk population target or rate | CWD prevalence (%) | Social welfare and components (millions of US$)† | | | |
|---|---|---|---|---|---|---|---|
| | | | | Social welfare | Harvest welfare | Feeding costs | Agricultural costs |
| Current (no CWD) | yes | 2,904 | 0.0 | 35.52 | 59.46 | 19.08 | 4.85 |
| FPT (no CWD) | yes | 4,500 | 0.0 | 39.32 | 75.21 | 28.90 | 6.99 |
| FPT (no CWD) | no | 3,100 | 0.0 | 39.32 | 46.43 | 0 | 7.11 |
| FPT | yes | 950 | 4.1 | 12.46 | 20.78 | 6.51 | 1.81 |
| FPT | no | 2,150 | 2.7 | 31.46 | 36.49 | 0 | 5.03 |
| FHR | yes | 26% | 13.8 | 13.73 | 20.04 | 4.96 | 1.35 |
| FHR | no | 17% | 4.4 | 31.92 | 36.92 | 0 | 5.00 |
| PTS | yes | 900; 1600 | 5.6 | 13.97 | 22.80 | 6.58 | 2.25 |
| PTS | no | 1,850; 2,400 | 4.1 | 32.19 | 37.13 | 0 | 4.94 |
| Spatial FPT‡ | yes | 250 | 26.0 | 4.08 | 4.85 | 0.49 | 0.28 |

*Notes:* FPT, fixed population targeting; FHR, fixed harvest rate; PTS, population target switching. Chronic wasting disease (CWD) prevalence is a trailing 12-month average at year 20.

†Social welfare is calculated as in Eq. 10, harvest welfare is the discounted value of hunting benefits less any necessary Wyoming Game & Fish depopulation costs, and other costs are expressed as discounted values. Agricultural costs include expected brucellosis quarantine costs, self-protection costs, government vaccination subsidies, and depredation costs.

‡The spatial FPT case shown in Table 1 is only one of the possible spatial configurations; all feedgrounds are closed except for Fall Creek, as shown in Fig. 4.

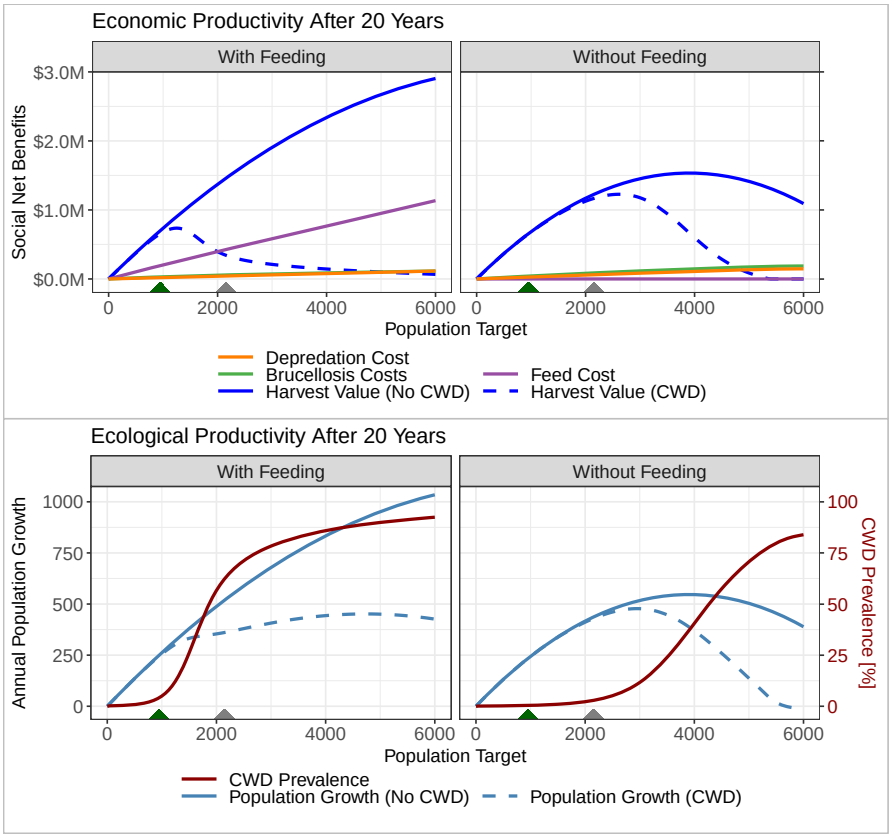


FIG. 3. Ecological and economic productivity at year 20 for various elk population targets under the fixed population target (FPT) management practice. Population growth, chronic wasting disease (CWD) prevalence, and annual economic values at year 20 ($t = 20$) are plotted as functions of the population target. Values are shown for two scenarios: one in which feedgrounds remain open and one in which feedgrounds are closed starting at time $t = 0$. The top plots show the economic data series. The bottom plots show the ecological data series. The dark green and gray triangles along the horizontal axes depict the optimal population targets with and without feeding.

feeding is discontinued. This is because the productivity impacts of CWD are smaller when there is no feeding (Fig. 3), and so, there are fewer economic benefits to adjusting the population target in this case. The reduction of agricultural costs (29%) is also modest given the relatively small costs arising in the CWD-free case.

An important difference between the CWD-free and CWD scenarios is that the elk population target is much larger with feeding in the CWD-free case, whereas the target is smaller with feeding in the presence of CWD. In the CWD-free case, feeding provides significant hunting benefits with a comparatively small increase in brucellosis costs. In contrast, when CWD is present, feeding imposes a significant cost to hunters because the congregation of elk at feedgrounds increases CWD prevalence. As a result, managers may wish to consider discontinuing feeding in the presence of CWD, although ranchers experience greater costs in this case. The larger brucellosis and depredation costs arise because in the short term, feedground closures spur elk movement to private lands. While the long-term costs of brucellosis eventually diminish, the short-term costs are weighed more heavily due to discounting. These results indicate that, under the FPT scenario, hunters switch their preferences about feeding in response to CWD risks and associated elk population targets: hunters prefer feeding without CWD risks, as might be expected, but the opposite is true when there are CWD risks.

### *Fixed harvest rate*

The second strategy considered is harvesting a fixed percentage of the elk population each year. This strategy allows depopulation to occur more gradually than under the FPT strategy. First consider the case where feeding is continued. The welfare-maximizing fixed harvest rate is 26%, and social welfare is 10% higher than the FPT strategy with feeding. In contrast, we found (not reported in Table 1) the FPT strategy to be preferred when there are no limits on elk hunting and thus no agency costs. This result indicates that, with limits on the number of elk that can be hunted within a year, it is better to achieve elk population reductions gradually via the FHR strategy because discounted agency

depopulation costs are reduced when they are spread over a slightly longer time horizon.

The optimal fixed harvest rate declines to 17% if feeding is discontinued, with social welfare only slightly higher than that arising under the optimal FPT strategy. The small welfare difference indicates that the costs of gradual depopulation in terms of increased disease transmission and reduced ecological productivity are approximately offset by the benefits of avoiding agency depopulation costs. The primary difference between the FPT and FHR practices is that CWD prevalence initially spikes to 21% in the FHR feeding case because the elk population cannot be reduced as quickly in this case, but hunting alone is able to achieve the desired elk population over time.

### *Population target switching*

The population target switching management strategy (PTS) with $\varepsilon = 4.0\%$ yields surprising results. We expected the flexibility of allowing the population target to increase following the reduced prevalence of the disease would lead to an improvement in welfare. However, adding this additional management flexibility did not produce substantial welfare gains relative to having a single population target. PTS does not improve welfare estimates much because the added management flexibility is limited (e.g., relative to a time-varying population target) and populations that are above 950 elk with feeding (which is the corresponding target in the FTP case) or 2,150 elk without feeding quickly lead to higher CWD prevalence levels. Such an increase in CWD prevalence triggers a decrease in the population target under the PTS strategy. The benefit associated with a brief increase in the population target (and elk productivity) is almost entirely offset by the cost of a CWD outbreak, so such an increase leads to only small economic gains.

### *Spatially strategic management*

One advantage of the spatial bioeconomic model is the ability to investigate management strategies that vary over space. Here we consider 14 combinations of hypothetical closures of different subsets of feedgrounds to see if strategically located supplemental feeding under FPT management with CWD can generate a level of social welfare similar to that under full termination of the supplemental feeding program. In the simulations, we use the same RSF coefficients and variables for the full feeding scenario, but adjust feeding levels and scale steady-state brucellosis prevalence ($\theta_{BRUC}$) down according to the percent of feedgrounds that are closed. The main finding from these simulations is that strategically closing a subset of existing feedgrounds results in an economic loss. The reason that closing certain feedgrounds (e.g., ones farther from an elk migration route or closer to private land) does not improve welfare is that elk will simply congregate more densely at the feedgrounds that remain open (see Fig. 4 for elk population densities for one of the possible combinations: one feedground is left open and three are closed). Because elk density will increase as elk disperse to the remaining open feedground(s), CWD will spread even more rapidly through the population and cause a sharp welfare loss to hunters. Since elk population management is determined jointly with CWD transmission and elk dispersal, the optimal management response is to greatly reduce the elk population target to limit the density-dependent spread of CWD. This is similar to the dispersal spillovers caused by the creation of protected areas (Sanchirico and Wilen 2001) and closing areas to harvests, although the spillovers in our case are negative due to higher species density and more rapid disease transmission.

## Discussion

A number of biologists, ecologists, and epidemiologists have expressed concerns about the consequences of continuing supplementary feeding of elk in the GYE, especially given the impending introduction of CWD. Using a spatially explicit bioeconomic model, our results suggest the continuation of feeding and current elk population management could result in present-value welfare losses of US$19 million if CWD is introduced for our case-study area. The welfare losses are likely to be larger for the entire GYE region. In contrast, for the hypothetical case where there is no risk of CWD being introduced into the study area, supplemental feeding along with adapted harvest management would provide the highest social welfare, including to the benefit of both ranchers and the hunting industry. The results differ because of the economically optimal elk management response to CWD risks. Specifically, elk management responses to CWD risks result in much lower elk population targets to reduce density-dependent and environmental transmission of CWD. As feeding fuels CWD risks, the targets would have to be even lower, with significantly lower benefits to hunters, when feeding occurs. As feeding is also an expensive practice in its own right (Dean 1980, Boroff 2013, Boroff et al. 2016), it is better from an economic perspective to eliminate feeding and increase population targets relative to the targets with feedgrounds.

The benefits and costs of elk management in response to CWD risks accrue differently to hunters and ranchers. Discontinuing feeding will, especially in the first year, increase brucellosis and depredation costs for ranchers associated with elk using private lands. However, our model predicts that these costs are outweighed by the economic benefits to hunters, guides, outfitters, and other regional businesses that provide goods and services to hunters. These benefits accrue to a relatively large and diffuse number of people, whereas the increased brucellosis-related costs fall on a relatively small number of local ranchers. Economic theory suggests that a system could be devised wherein those who gain from

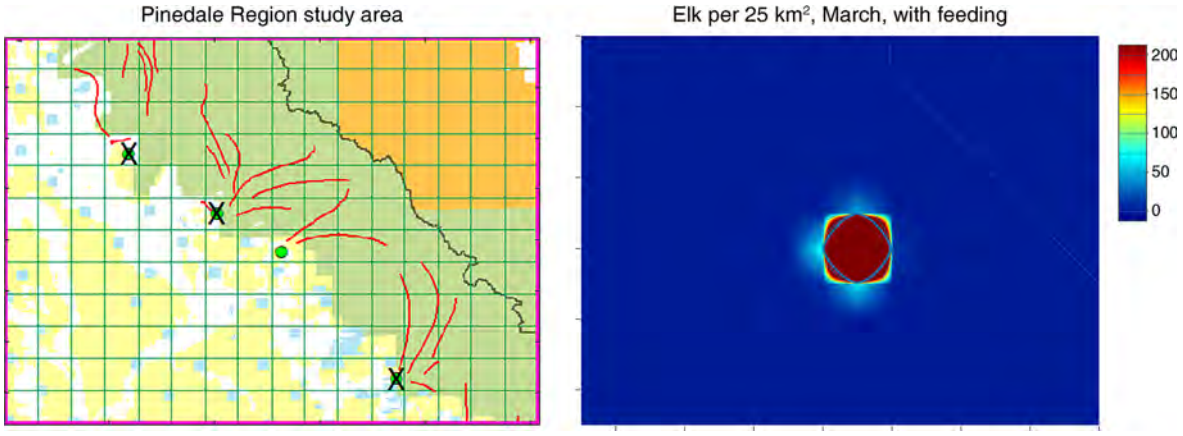


FIG. 4. Elk population density heat map when Fall Creek feedground remains open. The left graph is copied from Fig. 1 with an "X" through feedgrounds that are closed in the model simulations. The remaining open feedground is Fall Creek. The graph on the right shows a heat map of elk density around the Fall Creek feedground during March.

discontinuing feeding in response to CWD could compensate those who lose. Compensation could, for example, help ranchers increase self-protection and mitigate depredation as feeding is discontinued. Eventually, the need for increased self-protection against brucellosis, and a potential role for compensation, should dissipate as the prevalence in the elk population falls over time in the absence of feeding. Note that, since aggregate economic welfare is maximized in our neutral cost–benefit analysis, post-compensation outcomes can leave all stakeholders better off than when policies are driven by analyses that weight stakeholder groups unevenly (e.g., based on political power considerations).

One possible limitation to our analysis is that brucellosis costs to ranchers are uncertain, particularly if feedgrounds are closed. Potential additional costs may include larger brucellosis infection risks to cattle (i.e., greater probability or cost of infection), or behavioral responses to mitigate this risk such as having to transport cattle outside of the area during the transmission risk period to keep elk from comingling with cattle. To assess the worst-case scenario for ranchers, we force all ranchers to invest in elk-proof fence around their winter pasture and delay grazing on public land until the risk of brucellosis transmission is negligible. It is equivalent to setting the self-protection intensity to $\Phi_{i,t} = 1$ for all ranchers. Under this FPT scenario, the welfare gap between supplemental feeding (−US$179.8 million) and no supplemental feeding (−US$158.8 million) is US$21.0 million, which is similar to the case where ranchers choose the level of brucellosis self-protection. Full protection is very expensive for ranchers (hence the negative net welfare values) and not the preferred option, yet the analysis still indicates that discontinuing feeding is economically optimal. This, along with the consistent findings from the sensitivity analysis in Appendix S1, suggests that our main result, the costs of continuing to feed elk after the introduction of CWD outweigh the benefits, is robust.

We close by discussing some possible extensions to the analysis. First, it might be interesting to examine whether attempts to manage elk age and sex distributions could improve the efficiency and effectiveness of disease management (Fenichel and Horan 2007a). Second, if wolves enter the study area, they could be incorporated into the model to factor in their current and future impact on the elk population, livestock, and disease dynamics under alternative strategies for managing CWD and brucellosis. Wild et al. (2011) proposes that wolves may act as a natural disease control mechanism in deer by eliminating infected, weak individuals from the population. Assuming a similar mechanism occurs with elk, disease control may be an unrecognized ecosystem service benefit generated by wolf populations. Third, recent research has shown that some members of the elk population show greater susceptibility to CWD than others due to genetic variation (Williams et al 2014). Over the time horizon considered in our simulations, a significant shift in the genetic makeup of the population might occur as elk with the more favorable genotype survive and reproduce more effectively (O'Rourke et al. 1999, Monello et al. 2017). A fourth extension would be to use better data, if and when it becomes available, to more accurately calibrate the environmental contamination and transmission processes for CWD. Lastly, the presence of elk in the GYE is known to provide value to the local economy by drawing wildlife viewers. However, for our case study, we assume wildlife viewing and tourism benefits can be reasonably excluded from the model because tourists interested in viewing wildlife typically travel farther north to the National Elk Refuge or Yellowstone National Park. That said, our model does not account for welfare losses arising from local residents or visitors having to watch elk suffer from either the effects

of CWD or inadequate feed resources in the absence of feedgrounds, particularly during severe winters. Such costs and benefits will need to be added in the future if this model is applied to other areas in the GYE where wildlife viewing tourism is more significant.

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## Supporting Information

Additional supporting information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/eap.2129/full

## Data Availability

The input data sets required to run the simulations shown in this paper, including the RSF regression coefficients but excluding the elk telemetry data used to estimate the coefficients, are openly available in Figshare at https://doi.org/10.6084/m9.figshare.11862810. The elk telemetry data set used to estimate the RSF coefficients is available from ScienceBase at https://doi.org/10.5066/f7474803.

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# Comparison of 2 ELISAs for detecting exposure to *Brucella ovis*

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**Abstract.** Control of *Brucella ovis* infection in sheep flocks in the United States depends on early detection of *B. ovis* antibodies via serologic testing. We used 2,276 sheep sera and various cutoff values to compare seroprevalence and agreement between 2 ELISAs: the National Veterinary Services Laboratories (NVSL) *B. ovis* indirect ELISA and the IDEXX *B. ovis* ELISA kit. A subset of 295 sera was used to compare agreement and evaluate relative sensitivity and specificity of the 2 ELISAs with an agar gel immunodiffusion (AGID) test kit. There was no significant difference in *B. ovis* seroprevalence between the ELISAs; however, there was poor agreement between them. When the AGID test was used as the reference test, the IDEXX ELISA with a moderate cutoff value (S/P ratio = 45%) had the highest relative sensitivity of 38.1% and specificity of 92.0%. The NVSL ELISA with a lax cutoff value (S/P ratio = 0.75) had relative sensitivity of 19.1% and specificity of 94.6%. Receiver operating characteristic analysis revealed that optimal cutoff values for the NVSL and IDEXX ELISAs were 0.091 and 16.5%, respectively. This results in sensitivity and specificity of 85.7% and 31.8% for the NVSL ELISA, and sensitivity and specificity of 81.0% and 53.6% for the IDEXX ELISA, respectively.

**Key words:** agar gel immunodiffusion; *Brucella ovis*; ELISA; ram epididymitis; sheep.

*Brucella ovis* is the causative agent of a sexually transmitted, infectious disease that causes clinical or subclinical chronic disease in domestic sheep.[13] Ovine brucellosis is characterized by genital lesions and epididymitis in rams, placentitis and rare abortions in ewes, and premature lambs or lambs with low birth weights.[3,10,13] Interference with serologic tests by vaccines limits the use of a vaccine in some countries, and antimicrobial treatment is not financially feasible; hence, a test-and-slaughter management strategy is recommended for eliminating *B. ovis* infection in flocks in the United States.[1]

Detection of *B. ovis* exposure depends mainly on serologic testing; the complement fixation test (CFT), agar gel immunodiffusion (AGID) test, and ELISAs are the primary assays used to detect *B. ovis* antibodies in serum.[1,13] In the United States, the National Veterinary Services Laboratories (NVSL) *B. ovis* indirect ELISA (APHIS-NVSL) is the only ELISA used for detection of *B. ovis* antibodies in sheep and goat serum.[13] Although the antigen and controls are purchased from NVSL, each diagnostic laboratory is responsible for buying reagents, coating microtiter plates with antigen, and running the assay according to protocol, resulting in discrepancies between laboratories. The NVSL ELISA has 2 cutoff values, which result in 3 categories: seropositive, seronegative, and indeterminate. The indeterminate result category is difficult to interpret and often requires extra sample collection for additional serologic testing. In contrast, the IDEXX *B. ovis* ELISA kit (IDEXX Laboratories) is a standardized commercial kit used for detection of *B. ovis* antibodies in sheep serum in Europe. The kit provides antigen-coated microtiter plates, controls, and reagents. Originally, the IDEXX ELISA had 2 cutoff values, which created 3 result categories: seropositive, seronegative, and suspect. The suspect result category was similar to the indeterminate result category for the NVSL ELISA; however, IDEXX developed 3 new, single cutoff values for producers and veterinarians to use depending on the epidemiologic situation of the flock and purpose of *B. ovis* testing.[9]

Our objective was to compare the NVSL and IDEXX ELISAs with each other and a commercial AGID test kit (TECPAR) to evaluate their potential for use in disease detection on U.S. sheep operations. This information could be utilized by veterinarians, diagnosticians, and producers to determine which assay is best suited for their needs.

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Between August 2015 and May 2016, sera were collected from 2,276 sheep from 18 operations in Wyoming. The University of Wyoming Institutional Animal Care and Use Committee (IACUC) approved the study and sampling method (protocol 200150622KS00178-01). Flocks varied in geographic location and size, and sheep varied in age, sex, and breed.[6] During sample collection, properly trained personnel drew 5–10 mL of blood via jugular venipuncture of healthy sheep using 10-mL blood collection tubes and needle holders (Vacutainer; Becton, Dickinson) with disposable 4-cm, 18–20-ga needles (Greiner Bio-One). Blood was allowed to clot before transportation back to the Wyoming State Veterinary Laboratory (Laramie, WY) on ice. Sera were separated from blood samples by centrifugation, decanted into labeled tubes, and stored at –20°C until serologic testing.

All samples were initially analyzed using the NVSL ELISA according to the manufacturer's instructions. The diagnostic sensitivity and specificity are reported to be 96.3% and 99.6%, respectively,[7] and the NVSL-supplied antigen was a Triton X-100 extract of the washed REO 198 strain. Controls run in triplicate included high positive, low positive, and negative; sample sera were run in duplicate. Polystyrene microtiter plates (Nunc) were coated with an antigen dilution in carbonate buffer, incubated, and blocked with bovine serum albumin solution (KPL). Conjugated protein G–biotin from *Streptococcus* spp. (MilliporeSigma), Vectastain conjugate (Vector Laboratories), blue phosphatase substrate (KPL), and APstop solution (KPL) were prepared according to the manufacturers' instructions. During the assay procedure, sera, conjugated protein G–biotin, and Vectastain conjugate were incubated at 37°C in a humidified chamber inside a digital display incubator (Boekel Industries) for 45 min, 35 min, and 25 min, respectively. After each incubation, plates were washed with a high-salt wash solution, excess liquid emptied in the sink, and plates were tapped onto absorbent material to remove any nonspecifically bound proteins or antibodies. The blue phosphatase substrate incubation step was performed at room temperature on a microplate shaker, and color development stopped when the low-positive control average reached an optical density (OD) between 0.350–0.500 at a wavelength of 620 nm on a microplate absorbance spectrophotometer (Tecan GENios Pro; BioTek Epoch Instruments). The average OD for each sample and control was calculated, and the average sample-to-positive (S/P) ratio used to determine sample status. Original S/P ratio cutoff values were as follows: $>0.75$ = positive, $\geq 0.40$ to $\leq 0.75$ = indeterminate, and $<0.40$ = negative. The following equation was used to calculate the S/P ratio:

$$\text{S/P ratio} = \frac{\text{OD of blanked sample average}}{\text{OD of low-positive control average}}$$

Sera were then tested using the IDEXX ELISA according to the manufacturer's instructions. The diagnostic sensitivity and specificity are reported to be 91.7% and 95.2%, respectively,[9] and the antigen used is an extract of the REO 198 strain prepared by IDEXX. Each kit contained 2 antigen-coated plates, positive and negative control sera, anti-ruminant IgG monoclonal antibody labeled with horseradish peroxidase as conjugate, tetramethylbenzidine (TMB) substrate, stop reagent, and wash solution. Controls and sample sera were run in duplicate. During the assay procedure, sera and conjugate were incubated at 37°C in a humidified chamber inside a digital display incubator (Boekel) for 60 min each. After each incubation, plates were washed 3 times with the wash solution, excess liquid emptied in the sink, and plates were tapped onto absorbent material. The TMB substrate incubation step was performed at room temperature for 15 min before adding the stop reagent. The plate was read at a wavelength of 450 nm on a microplate absorbance spectrophotometer (Tecan; BioTek Instruments), the average OD for each sample and control was calculated, and the average S/P ratio was used to determine sample status. Original S/P ratio cutoff values were as follows: $>50\%$ = positive, $\leq 50\%$ to $\geq 10\%$ = suspect, and $<10\%$ = negative. Suggested cutoff values of 30%, 45%, or 60% were also assessed.[9] The following equation was used to calculate the S/P ratio:

$$\text{S/P ratio} = \frac{\text{OD of blanked sample average} - \text{OD of blanked negative control average}}{\text{OD of blanked positive control average} - \text{OD of blanked negative control average}} \times 100\%$$

Finally, 295 sera were tested using the AGID test according to the manufacturer's instructions. These 295 sera were chosen based on initial results from the NVSL ELISA. Of these samples, 20 were seropositive and 46 were indeterminate. The remaining 229 seronegative samples were chosen randomly. The diagnostic sensitivity and specificity of the AGID test kit is reported to be 70.1% and 100%, respectively.[14] Each kit contained 1 vial of lyophilized antigen prepared from soluble proteins and lipopolysaccharides extracted from the *B. ovis* REO 198 strain prepared according to OIE international standards,[13] 3 vials of lyophilized positive control serum, and 1 vial of diluent. A predetermined dilution of 1% agarose solution in borate buffer (pH 8.3) was boiled in a water bath until the gel was translucent and homogeneous. Agar was poured into mini petri dishes, packed in a well-sealed humidified chamber, and stored in a refrigerator until serologic testing. Seven wells forming a hexagonal shape (1 central and 6 peripheral) were made, and agar was removed with a vacuum. Control and sample sera were distributed in peripheral wells alternating between positive control and sample sera, and the antigen was placed in the central well. Petri dishes were placed in a well-sealed humidified chamber, incubated at room temperature, and read after 48 h using an indirect light system with a black background. The presence of a precipitin line was considered a positive reaction.

Pairwise 2-tailed *z*-tests were performed to determine statistically significant differences in seroprevalence between the 2 ELISAs (Epitools epidemiological calculators; Ausvet, http://epitools.ausvet.com.au). A $p$ value of ≤0.05 was considered significant. Agreement between pairs of assays, including the AGID test, was estimated using the Cohen kappa coefficient (κ) and the overall proportion of agreement with 95% CIs (Epitools epidemiological calculators; VassarStats, http://vassarstats.net). We interpreted κ as follows: values ≤0 = no agreement, 0.01–0.20 = slight agreement, 0.21–0.40 = fair agreement, 0.41–0.60 = moderate agreement, 0.61–0.80 = substantial agreement, and 0.81–1.00 = almost perfect agreement.[5] Sensitivity and specificity of the ELISAs were calculated, and receiver-operating characteristic (ROC) analyses were performed (Epitools epidemiological calculators) using the AGID test results as the reference test. Few studies have estimated *B. ovis* prevalence in the United States; therefore, prevalence of 50% was used to place equal weight on the importance of both sensitivity and specificity. The area under the ROC curve (AUC) was calculated (Epitools epidemiological calculators), and AUC values were interpreted as follows: values of 0.5–0.7 = low assay accuracy, 0.7–0.9 = moderate assay accuracy, and 0.9–1.0 = high assay accuracy. Optimal cutoff values for maximizing test efficiency were determined using the Youden index (Epitools epidemiological calculators).

Of 2,276 sera, 20 (0.88%) were seropositive by NVSL ELISA, and 25 (1.10%) by IDEXX ELISA, according to original cutoff values. Seven (0.31%) were seropositive on both ELISAs, and 2,049 (90.03%) were seronegative on both ELISAs. When comparing seroprevalence, there was not a statistically significant difference between the NVSL and IDEXX ELISAs (pairwise 2-tailed *z*-test, $p = 0.4535$; Table 1).

Overall, there was poor agreement between different assay cutoffs utilized in this study, based on κ coefficients (Table 2). For the NVSL and IDEXX ELISAs, we first compared the original cutoff values, which included the indeterminate or suspect result. Then, we used multiple single cutoff values to compare ELISAs with each other and the AGID test. The cutoff values used for the NVSL ELISA included a lax and strict value (i.e., 0.75 and 0.40, respectively). The cutoff values used for the IDEXX ELISA included a lax, moderate, and strict value (i.e., 60%, 45%, and 30%, respectively). The lax cutoff values correspond with the higher threshold given the expected decrease in sensitivity caused by possible false-negative results. In contrast, the strict cutoff values correspond with the lower threshold given the expected increase in sensitivity caused by true-positive results. ELISA combinations that resulted in moderate κ coefficients included the NVSL and IDEXX ELISAs with strict cutoff values (0.40 and 30%, respectively; $\kappa = 0.41$) and lax cutoff values (0.75 and 60%, respectively; $\kappa = 0.43$).

When comparing results from each ELISA with results from the AGID test ($n = 295$), the IDEXX ELISA with a moderate cutoff value (45%) resulted in the highest κ coefficient of 0.25, and overall agreement of 88.1%. The κ coefficient decreased to 0.16 and overall agreement increased to 89.8% when the lax cutoff value (60%) for the IDEXX ELISA was compared with the AGID test. The NVSL ELISA with a lax cutoff value (0.75) and the AGID test resulted in a κ coefficient of 0.14 and overall agreement of 88.8%.

**Table 1.** Analysis of 2,276 sheep sera for *Brucella ovis* antibodies according to manufacturers' original cutoffs.

| ELISA* | No. positive | Seroprevalence (%) | 95% CI |
|---|---|---|---|
| NVSL | 20 | 0.88 | 0.57–1.35 |
| IDEXX | 25 | 1.10 | 0.75–1.62 |

* NVSL *B. ovis* indirect ELISA; IDEXX *B. ovis* ELISA kit.

Results from the AGID test ($n = 295$) were used as the reference test to determine sensitivity and specificity of each ELISA with various cutoff values (Table 3). The IDEXX ELISA with a moderate cutoff value (45%) had the highest combined sensitivity and specificity of 38.1% and 92.0%, respectively. When using the lax cutoff value (60%) for the IDEXX ELISA, sensitivity decreased to 19.1%, but specificity increased to 95.3%. For the NVSL ELISA, the lax cutoff value (0.75) resulted in the highest sensitivity and specificity of 19.1% and 94.6%, respectively.

ROC analysis revealed the average ELISA result for AGID-positive and AGID-negative animals, the AUC value, and the optimal cutoff value with reported sensitivity and specificity for each ELISA (Table 4). For the NVSL ELISA, the mean S/P ratio for AGID-positive animals was 0.448 (95% CI: 0.043–1.77; median: 0.236) and the mean S/P ratio for AGID-negative animals was 0.272 (95% CI: 0.027–0.895; median: 0.151). The AUC value was 0.584 (95% CI: 0.452–0.716), which indicates low assay accuracy when compared to the AGID test. Assuming prevalence is 50%, the recommended cutoff value for the NVSL ELISA is 0.091, resulting in sensitivity of 85.7% and specificity of 31.8%. When using the strict cutoff value for the NVSL ELISA (0.40), the sensitivity and specificity were 23.8% and 77.4%, respectively. For the IDEXX ELISA, the mean S/P ratio for AGID-positive animals was 37.7% (95% CI: 9.76–97.9%; median: 24.0%) and the mean S/P ratio for AGID-negative animals was 17.4% (95% CI: <0.001–56.5%; median: 15.5%). The AUC value was 0.704 (95% CI: 0.591–0.817), which indicates moderate assay accuracy when compared to the AGID test. Assuming prevalence is 50%, the recommended cutoff value for the IDEXX ELISA is 16.5%, which would result in a sensitivity of 81.0% and specificity of 53.6%. When using the moderate cutoff value for the IDEXX ELISA (45%), the sensitivity and specificity was 38.1% and 91.6%, respectively.

Our study demonstrated that the IDEXX ELISA detected *B. ovis* antibodies in more animals than the NVSL ELISA; however, the difference in seroprevalence between the 2

**Table 2.** Comparison of kappa coefficients and agreement for various assay combinations and cutoff values for *Brucella ovis* antibodies.

| Assay comparison* | Observed agreement† | κ† |
|---|---|---|
| NVSL (0.75) vs. IDEXX (60%) | 99.1 (98.6–99.4) | 0.43 (0.23–0.63) |
| NVSL (0.75) vs. IDEXX (45%) | 98.7 (98.1–99.1) | 0.39 (0.22–0.57) |
| NVSL (0.4) vs. IDEXX (45%) | 97.4 (96.6–98.0 | 0.36 (0.24–0.49) |
| NVSL (0.75) vs. IDEXX (30%)† | 97.4 (96.7–98.0) | 0.30 (0.17–0.42) |
| NVSL (0.4) vs. IDEXX (60%) | 97.4 (96.7–98.0) | 0.28 (0.15–0.41) |
| NVSL (0.4) vs. IDEXX (30%) | 96.7 (95.7–97.4) | 0.41 (0.30–0.52) |
| NVSL (0.75 and 0.4) vs. IDEXX (50% and 10%) | 91.2 (89.9–92.3) | 0.21 (0.15–0.28) |
| IDEXX (60%) vs. AGID | 89.8 (85.7–92.9) | 0.16 (0–0.34) |
| NVSL (0.75) vs. AGID | 88.8 (84.5–92.1) | 0.14 (0–0.31) |
| IDEXX (45%) vs. AGID | 88.1 (83.8–91.5) | 0.25 (0.08–0.42) |
| IDEXX (30%) vs. AGID | 76.3 (70.9–90.9) | 0.09 (0–0.20) |
| NVSL (0.4) vs. AGID | 73.9 (68.4–78.7) | 0.01 (0–0.10) |

* Numbers in parentheses are cutoff values.
† Numbers in parentheses are 95% CIs.

**Table 3.** Sensitivity and specificity for each ELISA with various cutoff values using AGID as the reference test ($n$ = 295).

| | AGID | | | |
|---|---|---|---|---|
| | + | – | Sensitivity | Specificity |
| NVSL (0.40 cutoff) | | | | |
| + | 5 | 61 | 23.8 (8.2–47.2) | 77.7 (72.3–82.5) |
| – | 16 | 213 | | |
| NVSL (0.75 cutoff) | | | | |
| + | 4 | 16 | 19.1 (5.5–41.9) | 94.2 (90.7–96.6) |
| – | 17 | 258 | | |
| IDEXX (30% cutoff) | | | | |
| + | 8 | 57 | 38.1 (18.1–61.6) | 79.2 (73.9–83.9) |
| – | 13 | 217 | | |
| IDEXX (45% cutoff) | | | | |
| + | 8 | 22 | 38.1 (18.1–61.6) | 92.0 (88.1–94.9) |
| – | 13 | 252 | | |
| IDEXX (60% cutoff) | | | | |
| + | 4 | 13 | 19.1 (5.5–41.9) | 95.3 (92.0–97.5) |
| – | 17 | 261 | | |

Numbers in parentheses are 95% CIs.

ELISAs was not significant. One explanation for the increased detection of seropositive animals with the IDEXX ELISA is that the assay is detecting low levels of *B. ovis* antibodies in sera, supporting the notion that the IDEXX ELISA is more sensitive. However, the increased detection of seropositive animals with the IDEXX ELISA could also be an artifact of cross-reactivity. Organisms such as *Dichelobacter nodosus* and *Corynebacterium pseudotuberculosis*, the causative agents for ovine footrot and caseous lymphadenitis, respectively, are present in Wyoming and may be responsible for some false-positive reactions in our sample subset.[1,2,4,11,12]

When we evaluated ELISA agreement, the highest percent agreement and κ coefficients were observed between the ELISAs with similar cutoff values. For example, the NVSL ELISA with its lax cutoff value of 0.75 and the IDEXX ELISA with its lax cutoff value of 60% yielded a κ value of 0.43. However, no pair of ELISAs resulted in a κ value equivalent to "substantial" agreement, which was unexpected. This suggests the transition from one ELISA to another may result in low intra-laboratory agreement. Specifically, a switch from the NVSL ELISA to the IDEXX ELISA with the original cutoff values would result in relatively low agreement. However, a switch from the NVSL ELISA to the IDEXX ELISA with new recommended cutoff values (i.e. 60%, 45%, or 30%) based on the purpose of testing or epidemiologic situation may be a better option.[9]

**Table 4.** Summary of receiver-operating characteristic (ROC) analysis with the average ELISA result for AGID-positive and AGID-negative animals, area under the ROC curve (AUC) values, and optimal and suggested cutoff values with reported sensitivity (Se) and specificity (Sp) for each ELISA.

| | NVSL | IDEXX |
|---|---|---|
| Average for seropositive | 0.448 (0.043–1.77) | 37.7% (9.76–97.9) |
| Average for seronegative | 0.272 (0.027–0.895) | 17.4% (<0.001–56.5) |
| AUC | 0.584 | 0.704 |
| Optimal cutoff value | 0.091 | 16.49% |
| | Se = 85.7%; Sp = 31.8% | Se = 81.0%; Sp = 53.6% |

Numbers in parentheses are 95% CIs.

The more important finding of our study resides in the agreement between the AGID and each ELISA with various cutoff values. The IDEXX ELISA with moderate and lax cutoff values and the AGID test resulted in the highest κ coefficients, suggesting that these cutoff values correctly identified the highest percent of AGID-positive and AGID-negative animals while taking into consideration the percent agreement based on random chance. Specifically, the IDEXX ELISA with the moderate cutoff value correctly identified 8 AGID-positive animals and 252 AGID-negative animals. The NVSL ELISA with a lax cutoff value correctly identified 4 AGID-positive animals and 258 AGID-negative ones. Based on these results, the IDEXX ELISA with the moderate cutoff value resulted in the fewest false-negative results (i.e., 13), while correctly identifying the highest number of true-positive results (i.e., 8) when the AGID is used as the reference test for this sample set. This moderate cutoff value is recommended in flocks that perform some serologic testing, but may possess seropositive or "suspect" animals, which are characteristics that apply to many Western flocks.[9]

When we used the AGID test as a reference test to calculate sensitivity and specificity, the IDEXX ELISA with the moderate cutoff value had the highest combined sensitivity and specificity. When the cutoff value was relaxed, both the sensitivity and specificity of the IDEXX ELISA decreased, but remained higher than the NVSL ELISA. The lax cutoff value of the NVSL ELISA resulted in lower sensitivity (19.1% from 23.8%), but higher specificity (94.2% from 77.7%) compared to the strict cutoff value, suggesting many of the indeterminate results are truly seronegative, not seropositive. If the strict cutoff value is used for the NVSL ELISA, there will be a large number (e.g., 61 in our study) of false-positive results, which may lead to the culling of truly negative animals. However, if the overall objective and disease management strategy of a particular flock is to eliminate all animals with *B. ovis* antibodies, the strict cutoff value will provide fewer false-negative results. False-negative results can be particularly detrimental when dealing with venereal diseases, including *B. ovis*, because 1 ram can breed 30–50 ewes in 1 breeding season.

When we looked at the optimal cutoff value for each ELISA, all recommended values were extremely stringent in order to increase diagnostic sensitivity. Although a cutoff value of 0.091 for the NVSL ELISA would result in higher sensitivity (85.7%), the specificity is only 31.8%. For the IDEXX ELISA, the recommended cutoff is 16.5%, resulting in a sensitivity and specificity of 81.0% and 53.6%, respectively. Given the low sensitivity values for both ELISAs, one concern is that false-negative results will likely be produced. False-negatives have implications for flocks that are trying to eliminate *B. ovis*, given that all seronegative animals may not truly be disease-free, and could be one reason for well-managed flocks to find seropositive animals when they are tested routinely. However, most flock owners are more concerned with disease-free animals testing as seropositive because this results in the culling of valuable animals. Thus, a follow-up test is recommended for any animal that tests positive for *B. ovis* antibodies. The preferred test would be one that has higher specificity, such as the AGID test. However, the commercial AGID test that we used is only available in Brazil. Another possible assay is PCR, given that it detects *B. ovis* DNA from semen or vaginal swabs; however, PCR could produce false-negative results as a result of intermittent bacterial shedding. Furthermore, we acknowledge that this would be impractical for many sheep producers, as these samples are much more difficult to obtain than blood samples.

Regarding potential bias and study flaws, sample handling and the decision to use the AGID test as a reference test may have influenced study outcomes. We included sera from 2,276 sheep on 18, non-random, producer-selected operations in Wyoming. Animals selected within the flocks often comprised the majority of a flock's breeding rams and a convenient subset of breeding ewes, resulting in a sample population that is not entirely representative of breeding sheep in the state. We created additional sample bias when we chose the 295 samples for AGID testing based on the initial results from the NVSL ELISA. Additional serologic testing to rule out cross-reactions from other organisms, including the causative agent of caseous lymphadenitis, *C. pseudotuberculosis*, was not performed. Another bias results from the length of time between sample collection and testing with each ELISA. We tested sera with the NVSL ELISA on average 33 d post-collection (95% CI: 32–35 d; median: 20 d). However, sera

were stored in a –20°C freezer for up to 10 mo before being tested with the IDEXX ELISA. Select sera were then sent to the Federal University of Paraná in Brazil to be tested with the TECPAR AGID test. Although the CFT is considered the gold standard test for *B. ovis*–free certification prior to international movement, we chose to use the AGID test as the reference test because it has comparable sensitivity and specificity to the CFT, but it is much simpler and less expensive to perform in non-specialized laboratories.[8,13,14] However, it is generally acknowledged that the AGID test is less sensitive and more specific, and this commercial AGID test kit has a published sensitivity and specificity of 70% and 100%, respectively.[14] For our study, it is possible that some infected animals, or "true positives," were not detected by the AGID, thereby artificially lowering the specificity estimates for the 2 ELISAs. Although much less likely, given an estimated 100% specificity, if the AGID misdiagnosed truly disease-free animals as infected, the number of false-negative results on the ELISAs would be inflated, contributing to artificially lowered sensitivity calculations. The only way to resolve this issue would be to perform a controlled study with the known infection status of animals and independently calculate sensitivity and specificity of each assay. Because there are limited studies available on assay performance for *B. ovis*, we feel it is important to understand the strengths and limitations of the currently available assays. This information should lead to improved management plans of sheep flocks worldwide.

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## Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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REVIEW ARTICLE

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# Comparisons of brucellosis between human and veterinary medicine

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**ABSTRACT**

Brucellosis is the world's most widespread zoonosis, but also ranks as one of the seven most neglected diseases, according to the World Health Organization. Additionally, it is recognized as the world's most common laboratory-acquired infection. There are a reported 500,000 incident cases of human brucellosis per year. However, true incidence is estimated to be 5,000,000 to 12,500,000 cases annually. Once diagnosed, focus is directed at treating individual patients with antibiotic regimes, yet overall neglecting the animal reservoir of disease. Countries with the highest incidence of human brucellosis are Syria (1,603.4 cases per 1,000,000 individuals), Mongolia (391.0), and Tajikistan (211.9). Surveillance on animal populations is lacking in many developed and developing countries. According to the World Animal Health Information Database, Mexico had the largest number of reported outbreaks, 5,514 in 2014. Mexico is followed by China (2,138), Greece (1,268), and Brazil (1,142). The majority of these outbreaks is *Brucella abortus*, the etiologic agent of bovine brucellosis. Brucellosis is an ancient disease that still plagues the world. There are still knowledge gaps and a need for better diagnostics and vaccines to make inroads towards control and eradication.

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## Introduction

*Brucella spp*., the etiologic agents of brucellosis, are Gram-negative, non-motile, facultative intracellular coccobacilli that can infect a wide range of mammalian species, including humans, and some amphibians [1,2]. There are 12 named *Brucella* spp., and four unnamed isolates (Table 1). *Brucella spp*. can be traced back 2.8 million years by presumptive evidence of pathologic changes in a late Pliocene hominin skeleton [3]. Additionally, molecular tests demonstrated the presence of *B. melitensis* DNA in a 700-year-old skeleton from medieval Italy [4]. The first description of the causative agent of brucellosis was made by Sir David Bruce in 1887 from the liver of a deceased solider on the island of Malta [5]. It was then termed *Micrococcus melitensis* [5]. Ten-years later, Bernard Bang isolated *Bacillus abortus* [6]. In honoring Sir Bruce, genus-nomenclature was standardized to *Brucella melitensis* and *Brucella abortus*, respectively [7]. Clinical human and animal brucellosis carries a plethora of synonyms including: undulant fever, Malta fever, Mediterranean fever, contagious abortion, Bang's disease, Neapolitan fever, Crimean fever, and Corps disease [8]. A majority of these names are still used in varying parts of the world.

Brucellosis is the world's most widespread zoonosis, but ranks as one of the seven most neglected diseases, according to the World Health Organization (WHO) [9,10]. There are approximately 500,000 reported incident cases of human brucellosis annually; however, true incidence is estimated at 5,000,000 to 12,500,000 cases annually [11–13].

Brucellosis is recognized as the world's most common laboratory-acquired infection [14]. This is attributed to the low infectious dose, estimated between 10–100 bacterial cells by aerosol or subcutaneous route [15,16]. In the developing world, *B. abortus, B. melitensis* and *B. suis* are leading causes of animal and human brucellosis [17]. However, with the recent identification of novel strains of brucellae, the complete picture of animal and human health is still unknown. The geographical distribution is changing with brucellosis re-emerging in some areas. Consistent case-reports of animal and human brucellosis originate from all continents with exception of Antarctica, in which only animals have tested positive [9,18,19].

Although brucellosis is the most widespread zoonosis worldwide, it remains severely neglected as a potential cause for chronic, debilitating maladies, due to its non-descript clinical presentation in human populations. This leads to major economic ramifications due to the loss of normal daily activities [9]. Diagnoses are challenging in areas with endemic malaria due to wide ranging clinical presentations [20]. Once diagnosed, focus is directed at treating individual patients with antibiotic regimens, yet

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**Table 1.** *Brucella* species by host. Zoonotic potential is classified as pathogenicity and virulence in human hosts. Original citation indicates the original publication where the species was characterized.

| Species | Natural host | Zoonotic Potential [8] | Original Citation |
|---|---|---|---|
| *B. melitensis* | Sheep, goats, and camels | Yes – High | [5] |
| *B. abortus* | Cattle, elk, and bison | Yes – High | [6] |
| *B. suis* | Pigs, hare, reindeer/caribou | Yes – High | [122] |
| *B. canis* | Dogs (domestic and wild) | Yes – Moderate | [123] |
| *B. ovis* | Sheep | No reported infections | [124] |
| *B. neotomae* | Desert wood rats | No reported infections | [125] |
| *B. ceti* | Cetaceans | Yes – Low | [126] |
| *B. pinnipedialis* | Pinnipeds | Yes – Low | |
| *B. microti* | Red foxes and common voles | No reported infections | [127] |
| *B. inopinata* | Unknown | Yes – High | [2,128] |
| *B. papionis* | Non-Human Primates | No reported infections | [129,130] |
| *B. vulpis* | Red fox | No reported infections | [131,132] |
| *Brucella NFXXXX* | Australian rat | No reported infections | [133,134] |
| *B. unnamed* | Blue dotted ray | No reported infections | [131] |
| *B. inopinata-like 09RB8471* | African bullfrogs and Big-eyed tree frog | No reported infections | [2,135] |
| *Brucella UK8/14* | White's tree frog | No reported infections | [136] |

overall neglecting the animal reservoir of disease. A cornerstone of zoonotic infectious disease epidemiology is the One Health concept. The goal of One Health is to employ a multidisciplinary approach to achieve the best health for people, animals, and the environment [21]. The control or eradication of the disease in wildlife and agricultural animals is a prerequisite for the control of the disease in human populations [22].

The aim of this review is to provide updated information on the global presence of disease, describe pathogenesis, risk factors, and clinical presentation in both humans and animals, describe potential control strategies, and to outline current and forthcoming diagnostics.

## Global presence of disease

Although the most widespread zoonosis, brucellosis is classified as a 'rare disease' by USA (U.S.) National Institutes of Health. This denotation is applied to most developed countries where incidence is low (USA: 0.40 cases per 1,000,000) [23]. Currently, the U.S. typically sees less than 100 reported cases per year, with most occurring in the south and southwest from illegally imported soft cheeses (unpasteurized) from Mexico [24]. However, in the U.S. true incidence has been estimated at five to 12 times greater, purely from foodborne illness [25]. Syria has been reported to have the highest incidence (1,603.4 cases per 1,000,000 individuals) of any country that report statistics to the WHO [26]. This is followed by Mongolia (3910), Iraq (268.8), Tajikistan (211.9), Saudi Arabia (149.5), and Iran (141.6) [23,26–28]. Several countries have had incidence above 200 in the past decade, but have since decreased dramatically, like Turkey (49.5) and Kyrgyzstan (88.0) [23]. A heat map of incidences is provided in Figure 1. Of note, many countries known to be endemic with human brucellosis are reported as 'no data.' This is due to the lack of surveillance and reporting to the WHO as well as the lack of peer-reviewed publications elucidating the incidence of disease. Conversely, the European Union has granted brucellosis-free status to many countries and human cases of brucellosis may have been travel-acquired and thus over-represent the national incidences of disease [26].

Utilizing the World Animal Health Information Database Interface (WAHIS; http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home) a datasheet was compiled to evaluate the number of animal brucellosis cases for *B. abortus, B. melitensis*, and *B. suis*. This interface has the drawback of voluntary reporting into the World Animal Health Organization (OIE) and therefore, suffers from reporting bias. Furthermore, those countries with the financial resources for surveillance are over-represented in this dataset. However, it is the only complete database to compile these statistics. For the calendar year of 2014 (the most recent year with complete information), Mexico had a total number of 5,514 reported outbreak events of brucellosis, 5,174 from *B. abortus*, 340 from *B. melitensis*, and zero from *B. suis*. Other significant countries were China 2,138 (2126, 0, 12), Greece 1,268 (269, 999, 0), and Brazil 1,142 (1142, 0, 0). A heat map of number of reported outbreaks in livestock from *B. abortus, B. melitensis*, and *B. suis* is provided in Figure 2.

## Pathogenesis, clinical presentation, and risk factor in humans

Brucellae can gain entry into the human host via inhalation, ingestion, contact with mucosa, or puncture wounds such as needle sticks [29]. This is followed by an incubation of 10–21 days (but as long as 12 months), a brief bacteremia, and localization to the mononuclear phagocyte system [30]. The parasitic intracellular niche of *Brucella* helps to limit the exposure to the host immune (innate and adaptive) responses and provide protection from antimicrobials [31]. There are two forms of brucellosis; acute and chronic. Untreated, infections can result in undulating fevers due to re-

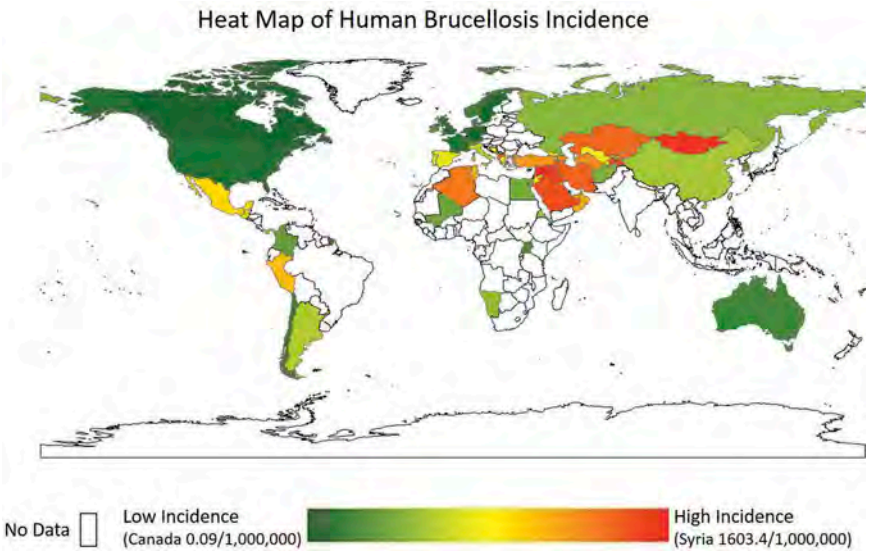


**Figure 1.** Heat map of human incidence (per 1,000,000 individuals). White space indicates no data. Adapted from Pappas et al., 2006 and other sources [23,27,28].

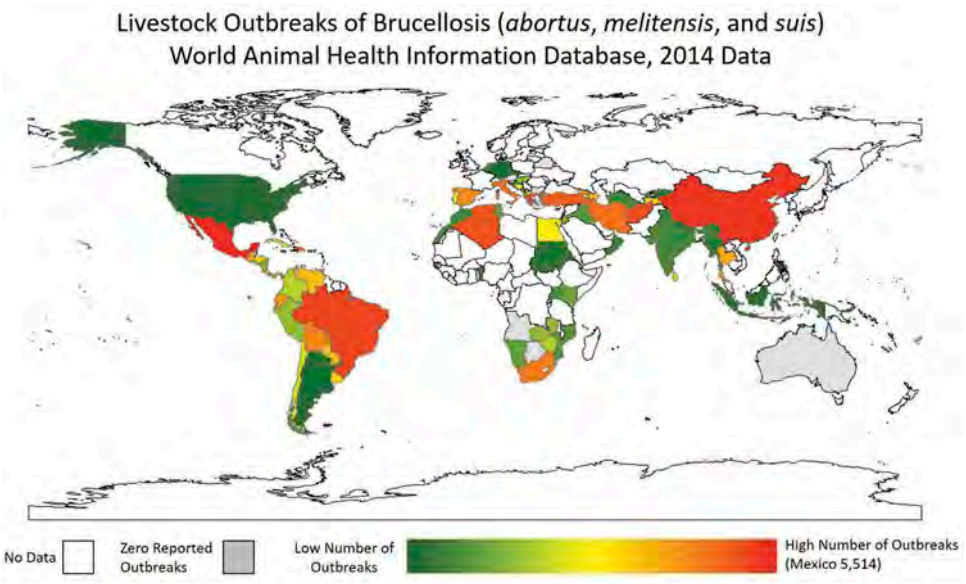


**Figure 2.** Heat map of number of brucellosis outbreaks (*B. abortus, B. melitensis*, and *B. suis*) in livestock as reported to WAHIS for the last complete year of data, 2014. White space indicates no data. Grey space indicates zero reported outbreaks.

current bacteremic episodes which is followed by new foci of infection (spine, joins, nerve, etc.). Humans, typically, do not produce clinical abortions due to brucellosis infections, thus constituting a dead-end host [29]. Abortions are a primary driver of transmission in animal populations [9].

Differences between human and wildlife/livestock clinical presentation are vast and there are significant differences in diagnostics and treatment strategies for the disease between species. Since humans are able to report symptoms, human brucellosis typically presents with arthralgia, pyrexia (undulant fever), and fatigue. In a retrospective analysis of 1,028 patients over a 10-year period in Turkey, it was found that gender differences were minimal as to the number of cases (female 52.4% vs. male 47.6%) where the mean age of cases was 33.7 ± 16.34 years [32]. Almost 70% of cases were between the ages of 13–44. Arthralgia was the most common reported symptom (73.7% of cases) followed by pyrexia (72.2%), fatigue (71.2%), hyperhidrosis (64.8%), and inappetence (49%). Clinical signs that were significant upon examination were pyrexia (28.8% of cases), hepatomegaly (20.6%), splenomegaly (14.5%), peripheral arthritis (14.3%), and hepatosplenomegaly (10.3%). Laboratory findings often included erythrocyte sedimentation rate elevation > 20 mm/h (59.9%), C-reactive protein positive (58.4%), anemia (40.3%), and transaminase elevation as defined by alanine and aspartate aminotransferase ≥ 50 IU/L (24.8%).

Human case-fatality proportion is very low; < 1% of clinical cases [33,34]. In one study, only five deaths out of 1,028 cases were reported [32]. The major predictor of death was the development of endocarditis. Incidence of endocarditis is around 2% of clinical cases, but responsible for 80% of the fatalities for brucellosis [23]. Cases typically present with chest tightness and shortness of breath combined with fever and fatigue [35]. In a cohort of 10 brucellosis-related endocarditis cases in China, six patients opted for valve replacement surgery and long-term anticoagulation drug regimens. These six cases were followed up for two to three years with a good prognosis. Four patients did not undergo valvular replacement and succumbed to their cardiac-related injuries within one-year of diagnosis [35]. All patients underwent antimicrobial therapies as recommended by the WHO [9]. However, difficulty arises with antimicrobial therapy for infective endocarditis patients in maintaining bactericidal concentrations of antibiotics at the site of bacterial colonization. Furthermore, due to non-descript clinical presentation of brucellosis, diagnosis may be delayed which will provide the bacterium sufficient time to progress to valve damage in these patients. Thus, the recommendation for these patients is continued antimicrobial therapy with replacement of the damaged valves [9]. Consideration should be given to length of therapy in these patients, as extended antimicrobial therapy may be warranted.

Another significant, albeit rare complication of brucellosis is neurobrucellosis. Intracellular invasion of the central nervous system occurs in about 5% of human clinical brucellosis cases [9]. The result of this invasion can be the development of meningitis, meningoencephalitis, brain or epidural abscesses, and/or demyelination disorders [36]. However, even with clinical neurobrucellosis patients, bacterial culture of the cerebral spinal fluid typically results in no growth of the organism [37]. A secondary complication of neurobrucellosis is ophthalmic brucellosis by affecting the optic nerve, either by inflammation or flow change of the optic nerve due to axonal degeneration [38]. Pathogenesis of neurobrucellosis is not well characterized. The vast majority of publications relate to case reports and case series [39].

The majority of clinical complications are osteoarthricular and can occur in 40% of clinical brucellosis cases [9]. The most common osteoarthricular malady reported is peripheral arthritis, typically affecting a single joint [9,40]. Only, 9% of peripheral arthritis cases are found bilaterally or are considered polyarthritis [40]. The second most common osteoarthricular complication is sacroilitis with secondary sciatica [9,41]. Other maladies include spondylitis, peripheral arthritis, bursitis, tenosynovitis, and rarely osteomyelitis. It is more likely to see radiologic evidence and complaints originating from the lumbar vertebrae rather than thoracic or cervical vertebrae [42]. Not surprisingly, those presenting with osteoarthricular brucellosis are more likely to have an elevated erythrocyte sedimentation rate than those without osteoarthricular brucellosis. However, there appears to be no statistical difference between groups with regard to C-reactive protein [40]. Therapeutic failure is three-times higher in osteoarthricular brucellosis compared to brucellosis cases without osteoarthricular complaints [40,43,44].

Genitourinary complications are seen in both humans and animals. In males, orchitis and epididymitis are most frequently reported and account for 6–8% of complications reported [9,32,45]. In females, pelvic abscesses and salpingitis are reported, albeit rarely [46]. However, in human populations there appears to be increased risk of fetal death in women with concurrent brucellosis infections. This association is disputed in peer-reviewed literature [47,48]. However, multiple studies hold that there is a true association between brucellosis infection and spontaneous abortions and fetal deaths [47,49–52]. This association could be explained by maternal toxemia, disseminated intravascular coagulation, or simply bacteremia. In one such study, a group of *Brucella*-seropositive pregnant women were matched against seronegative pregnant women. Spontaneous abortion and fetal death was statistically associated with seropositivity. However, there was no increased risk for preterm labor in brucellosis-infected mothers [49,51].

Risk factors for human brucellosis are limited to consumption of unpasteurized dairy products and occupational exposure. In one such study in Iran, consumers of unpasteurized dairy products had a 3.7 increased odds (95% CI 1.64–8.3) of developing brucellosis compared to controls [53]. Interestingly, this risk for transmission can be decreased (OR: 0.44; 95% CI 0.23–0.85) if individuals are aware of the risk. In a study from Tanzania, occupational risk factors include being an abattoir worker (OR: 7.87; 95% CI 1.42–57.25), presence on the slaughter floor (OR: 5.74; 95% CI 1.25–25.22), and cleaning of the facility (OR: 7.10; 95% CI 1.51–32.05) [54]. In endemic areas of the U.S., high risk occupational groups are National Park Service employees (Prevalence Ratio 3.9; 95% CI 1.50–7.27) and veterinarians (PR 2.5; 95% CI 1.30–4.68) [55];. Use of vaccines, specifically *B. abortus* Strain 19 (S19) had a statistically significant association with anti-*Brucella* antibodies in this sero-survey (Prevalence Ratio 2.7; 95% CI 1.4–5.2).

## Pathogenesis, and clinical presentation in animals

Animal brucellosis infection can occur via multiple differing routes. The most common is via the gastrointestinal tract, but conjunctiva or inhalation are possible [22]. Then, bacterium can translocate to lymphatic

vessels and gain access to the circulatory system and cause bacteremia. Tissue tropism includes pregnant uteri, male genital organs, mammary glands, and associated supramammary lymph nodes [22].

Different from human brucellosis, spontaneous abortion in infected ruminants is the hallmark of infection [56]. One of the contrasting differences between species is the presence of the carbohydrate erythritol, which plays a significant role in this clinical presentation in animals [57]. Erythritol is produced by placental tissue of species-specific pregnant animals and can be utilized by brucellae as a growth-stimulatory factor and carbon source, and is preferred over glucose [58]. Release of erythritol from the placenta into the circulatory system causes translocation of brucellae out of lymph nodes and migration to reproductive tissues. The new focus of infection is invasion of the chorionic villi, extending into the cotyledons on the fetal side of the placenta [59,60]. There, bacterium can replicate to a very high level ($10^{13}$ bacteria/gram of tissue) and induce infiltration of inflammatory cells, necrosis of trophoblast, and lead to vasculitis [61,62]. This ultimately leads to compromised fetal-maternal metabolic exchanges, resulting in fetal loss [59]. Fetal and placental tissues and associated fluids expelled in abortion events are the main transmission in animal populations [56]. The bacterium can reside in the environment up to a year, depending on the favorability of conditions (humidity, soil composition, temperature, ultra violet exposure, etc.) [63]. However, presence of scavengers can reduce the time of brucellae in the environment [64,65]. Of note, it has not been recognized that scavengers increase the risk of transmission to livestock, and it is generally believed that scavengers reduce risk of transmission [65]. Should an abortion event not take place, vertical transmission to offspring is still possible to perpetuate the infection. Mammary glands are a target organ for brucellae and secretion of viable bacterial cells through colostrum or milk is another important route of infection. This route is critical in human infections with the consumption of unpasteurized dairy products from infected animals.

The clinical presentation in animal populations largely varies depending on host species. Overall, in bovine brucellosis (*B. abortus*), caprine brucellosis (*B. melitensis*), and swine brucellosis (*B. suis*) animals can present with pyrexia (undulant fever), mastitis, weak offspring, spontaneous abortion, and carpal hygromas [66,67]. Spontaneous abortion is recognized as the cardinal sign of brucellosis infection. The bovine gestational period is approximately nine months. Typically, in bovine brucellosis, fetuses are aborted between the fifth and eighth month of gestation [18,67–69]. Infected pregnant cows or heifers will typically abort once, however, a subset will abort with future parturitions or birth weak calves [70]. That cattle typically only abort in the first pregnancy post-exposure is thought to be explained by acquired immunity after their first abortion event [63,67]. Mastitis is an important feature of the disease. Mammary glands as well as accessory lymph nodes are common niches for brucellae to replicate and evade immune defenses. *Brucella* bacteria are shed in the milk of infected, lactating cows. This is a secondary transmission route to naïve calves; however, it is the most important zoonotic transmission route to humans [12,26]. Infected bulls are thought to be a low risk of transmission to females, mainly due to the inhospitable environment of the vaginal tract [63,71]. Thus, while males can present with epididymis, orchitis, ampullitis, and seminal vesiculitis, it is believed that infected sperm in natural servicing is not a sufficient route of transmission. However, there is an appreciable risk in bull semen that is utilized in artificial insemination due to the intrauterine placement of the semen compared to vaginal deposition during natural servicing [72–74] . Semen from seropositive bulls has been found to contain *B. melitensis* [75]. In an experimental study of mature bulls, inoculation with *B. abortus* vaccine strain 19 led to the persistent shedding of the this bacteria in semen [76].

Swine brucellosis (*B. suis*) has the most wide-ranging clinical signs and is dependent on age, sex, exposure, and organ involvement [77]. Swine can present with abortion, birth of weak piglets, orchitis, epididymitis, infertility, arthritis, and lameness [67]. Pyrexia in swine is rare and not appreciated in the vast majority of cases. Unlike in cattle, sexual transmission of *B. suis* is the main source of transmission and can induce spontaneous abortions early in gestation [67]. Boars can present with appreciable genital infections, with unilateral testicular enlargement, which can result in infertility [67].

In small ruminants (sheep and goats), clinical signs of *B. melitensis* include abortion and weak offspring [9]. As with cattle, it is thought that abortion typically happens with the first gestation post-infection before acquired immunity can reduce the risk for future abortion events. However, there is still the possibility of future abortion events after the first parturition. Interestingly, in future pregnancies, infectious materials can be shed up to three months post-partum. In male sheep and goats, genital organs are the site of infection and can produce localized inflammation. This can lead to sexual transmission to naïve females in the flock.

*Brucella canis* infections in wild and domestic dogs have potential for zoonotic infections. Human infection with *B. canis* is usually asymptomatic or mild [67]. In canids, clinical signs are late-term abortion, mild pyrexia, and weak-litters. In male dogs, infection of the genital tract can result in epididymitis, orchitis, and prostatitis. Canids are able to clear the infection within two to three years [67]. Diagnostics of canine

brucellosis are lacking due to the phenotypic difference of *B. canis* [78].

## Control strategies

Control and eradication strategies vary between developed and developing countries. However, the burden of brucellosis infection is greatest in developing countries. Inconsistent infrastructure (animal health and pasteurization in particular) and lack of funding perpetuate the uncontrolled spread of disease [29]. In developed countries, like the U.S., great strides have been made since the initiation of and provision of funding for control and eradication efforts.

The greatest public health measure to impact zoonotic infections of brucellosis lies with pasteurization of dairy products. The pasteurization process kills microorganisms, like *Brucella spp*., that can potentially cause disease. However, even in developed countries (such as the vast majority of European countries, including those where brucellosis has not yet been eradicated in livestock populations) there is little to no restriction of raw milk and its products [79]. In fact, in the U.S., there is a raw milk movement and several states have passed legislation that allows the partially restricted sale of raw milk and raw milk products to consumers, mainly through a process of partial ownership in a communal animal. Recently, human cases of brucellosis were tied back to a raw milk dairy and implicated RB51 vaccine strain [80]. Many developing countries lack infrastructure to pasteurize dairy products prior to arriving to consumers [81]. In Tanzania, front-end cost of pasteurization facilities is not achievable at the current time and other control strategies are considered more economically feasible. In one study of 59 milk samples in Tanzania, 56% were culture positive for brucellosis [82].

A study conducted in Uganda was able to model a 47% decreased risk of human brucellosis if pasteurization could be implemented in the milk production chain [83]. Effective control programs in developing nations have a benefit to livestock, wildlife, and human populations. Finding a mechanism for funding via international aid as well as buy-in from public and private sectors would bear the best results in control and eradication [84].

## Vaccines

Concentrating control and eradication resources on livestock populations to control infections is typically accepted as the best method to manage brucellosis [12,84]. This can be achieved in one of several ways: vaccination, culling of infected animals, surveillance testing, or a combination of any of these. There is no vaccine that has been developed and approved for use in humans against brucellosis. However, in animal populations there are three main vaccines used for control. RB51 and S19 are directed at *B. abortus* infections in bovids, while Rev1 is used for *B. melitensis* in small ruminants [85]. While these vaccines do not prevent colonization and infection of animals, it decreases the likelihood of an abortion event, which in turn breaks the cycle of transmission and protects the remaining animals in the herd [84].

Vaccination is generally accepted as the most economically favorable measure for the control of animal brucellosis in endemic regions [86,87]. It is important to note that there are two colony morphologies to brucellae that provide background into brucellosis vaccines. One form is a smooth species that contains the smooth O-sidechain lipopolysaccharide (sLPS) [88]. Examples of smooth brucellae are *B. melitensis, B. abortus*, and *B. suis*. These outer membrane domains are recognized as the antigen by serologic assays. The second colony morphology of brucellae is the rough species that are deficient of the O-sidechain lipopolysaccharide (rLPS). Examples of rough brucellae are *B. canis* and *B. ovis*. Currently, there are two vaccines licensed for use in animal populations for *B. abortus*, one is a sLPS (S19), and the second is a rLPS (RB51), that is a smooth-strain mutant that lacks the O-sidechain lipopolysaccharide. A third vaccine, not currently licensed in the US, is a rLPS (S45/20). For *B. melitensis*, there is one vaccine that is licenses for use and is a sLPS (Rev1).

## Culling

In addition to vaccination, culling of suspect or reactor animals based on serology is used in most developed countries. The crux of this strategy relies on testing of herds to determine their sero-status. In developed countries, animals that test in the suspect or reactor range are removed from the herd and either sent to slaughter or are culled by regulatory officials for further definitive testing, such as bacterial culture. Many developing countries do not employ testing at the farm or in slaughterhouses to determine the status of the animals. This is primarily due to the lack of animal health infrastructure.

## Diagnostics

The primary class of diagnostics used in brucellosis surveillance is serologic testing in both humans and animals. There are various serologic tests that are based on the detection of either whole-cell antigen or the sLPS [89]. Overall, serologic tests are an ideal first line test. One major drawback are organisms that share the sLPS (*Yersinia enterocoloitica, Vibrio cholerae, Ochrobactrum anthropi, Salmonella enterica serotype Urbana, Franisella tularensis*, and *Escherichia coli*

*O157:H7*) and cross-react on these tests [90–92]. As with all serologic assays, presence of antibodies indicates exposure, but not necessarily present infection. The inception of serologic assays for brucellosis was in 1897 [93]. Since then assays have been improved and are currently offered in three general classes: agglutination tests; complement fixation tests; and primary binding assays.

Agglutination tests involve the addition of sample serum of unknown status to *Brucella* antigen and observing a pattern of agglutination in either a tube, microwell plates, or paper cards. Some current tests used in animal populations include the standard tube agglutination test (STA), acidified antigen (Rose-Bengal [RBT] or buffered antigen plate agglutination), 2-mercaptoethanol, rivanol (RIV), and the milk ring test. Depending on the assay, they can be relatively easy to perform (STA) or more labor intensive (RIV). Sensitivities are variable (as low as 21% in RBT), however, specificities are usually quite high (96.8–99.3%) [94–96].

Complement fixation tests (CFT) are typically used as a confirmatory test, which the USA Department of Agriculture (USDA) uses as a confirmatory assay on bovine samples. It relies on the presence of the $IgG_1$ isotype, which in turn will activate the complement cascade and lysis of an indicator (sheep red blood cells) will not take place. However, this assay is technically challenging and requires multiple reagents to complete, making adoption difficult in developing countries [89]. CFT requires subjectivity in reading test result. Nevertheless, the OIE has recommended this assay for use in international trade [89].

In addition to primary screening agglutination tests, primary binding assays like the fluorescence polarization assay are utilized in series or parallel in the U.S. Serum is used to measure the kinetics (spin) of molecules in solution. An unbound antigen that has a fluorescence marker will spin at a greater speed than an antibody-bound-antigen [97]. This measurement is taken in consideration with background emittance and produces a millipolarization value that can be used to classify an animal as negative, suspect, or reactor for brucellosis.

It is worth noting that replacement of serology is unlikely in the near future. Material cost is pennies per sample (USD), can be performed in minimal time, utilizes ante-mortem samples, and can be field deployable. However, in the U.S., an animal that tests positive via the diagnostic algorithm constructed by regulatory officials will need to be sampled post-mortem for definitive testing. This is completed with the gold-standard diagnostic test of culture. Drawbacks of culture are that it typically requires post-mortem samples from animals, can take up to 14-days, is costly (typically in the U.S., $600/animal USD exclusive of personnel costs), and suffers from imperfect sensitivity. In the U.S., only 30–50% of seropositive animals are culturable [98,99]. This leaves the question of the disposition of 50–70% of seropositive animals that are culture-negative. In the U. S., typically, 22–25 biologic samples are taken from serologically defined suspect or reactor animals. These samples are later plated on five media types in triplicate at U.S. federal labs. Additionally, growing of bacterial cultures provides the possibility of exposure and subsequent infection of laboratory personnel. Considering that brucellosis is the most common laboratory acquired infection in the world, risk to laboratory personnel is high. Therefore, care must be exercised in handling these cultures. *Brucella abortus, melitensis*, and *suis* are currently dually listed in the U.S. as Select Agents by the USA Department of Health and Human Services and the USDA [100]. This designation is given to microorganisms and toxins that have the 'potential to pose a severe threat to public health and safety' [100]. Therefore, it is not ideal to amplify live organism for diagnostics. In the U.S., upon culture confirmation of clinical samples, all biologic material must be either destroyed by an approved method or has to be moved to a biosafety level three laboratory or repository for future work. Ultimately, there is a lack of a true gold-standard test that is highly specific and sensitive for use in animal populations. Thus, a better diagnostic test is warranted.

There have been many efforts to develop molecular tests for the detection of brucellosis in post-mortem animal samples. Polymerase chain reaction (PCR) tests have proven both highly sensitive as well as highly specific in human clinical settings. Furthermore, PCR diagnostic testing can take a matter of hours from DNA extraction to results [101]. PCR has been used previously as a diagnostic test for animal brucellosis; however, the test has not been widely implemented due to lack of infrastructure and a wide range of sensitivity values [102]. The first *Brucella*-specific PCR was published in 1990 and was not species-specific [103]. It was validated on S19 vaccine isolates and not tested on spiked or field-collected samples. Additionally, it was unable to differentiate between species of *Brucella* and vaccine strains. The primers and amplicons were never published, so further analysis was not possible [104]. The next advancement in *Brucella* PCRs came in 1992 when primers were designed to amplify a region of the 16S rRNA [105]. Unfortunately, it also amplified the closest known relative to *Brucella, Ochrobactrum anthropi*. Again in 1992, a primer set targeting BCSP31 antigenic periplasmic protein of *B. abortus* was targeted [106]. However, it too amplified all *Brucella* species as well as *O. anthropi*. These primers targeting BCSP31 are still used today in diagnostics in the Middle East, Asia, and East Africa [107–110]. However, it does not exclude vaccine strains from detection and was validated only on post-culture colony isolates [111].

Laboratory results in human patients needs to be viewed with consideration of clinical findings, medical history, hematological testing, and radiographic findings. As stated previously, because humans are able to self-report symptoms, a patient will typically seek care upon an extended febrile episode. This is correlated with the bacteremia associated with a brucellosis infection [112]. Therefore, blood cultures are routinely performed in these patients. Availability of technologies, such as the automated continuously monitored blood culture systems, allows for growth of brucellae in a clinical sample. Additionally, bone marrow cultures can result in 15–20% higher yields of brucellae than peripheral blood cultures [113,114]. Cultures from patients with the acute disease can have sensitivities of 50% to 80%; while the chronic form is less likely (< 5%) to produce a culture [45,113,115]. However, a major drawback is that cultures must be incubated for six-weeks before reporting a negative result [116]. Culture maintains near 100% specificity as colonies will not grow if targeted bacteria is not present in the clinical sample.

Serologic assays are commonly utilized in brucellosis diagnostics and surveillance in human populations. Acidified-RBT-agglutination assays can be conducted similarly to animal diagnostics. However, serology can suffer from false-negative results in chronic cases of brucellosis [117]. Test statistics for RBT are 100% sensitivity and 97% specificity using a ≥ 1:1 cut-off value in acute cases of disease [118]. Of note, this cut-off value is not clinically meaningful as any dilution or mishandling of the sample may lead to false-negative result. Serum agglutination tests (SAT) are also used in human brucellosis diagnostics and utilize IgG, IgM, and IgA antibodies. Test statistics for SAT are 87.4% sensitivity and 100% specificity using a cutoff of ≥ 1:160 [118,119]. A smaller version of SAT is the microagglutination test (MAT) that can be performed in microtiter plates. This assay uses smaller volumes of serum and reagents and is appropriate for running multiple samples at the same time. MAT has the same test statistics as the SAT [118]. Both SAT and MAT have the same downfall of the inability of identifying chronic cases of the disease. Therefore, the cornerstone test for human and animal brucellosis is the enzyme-linked immunosorbent assay (ELISA). The popularity of the ELISA assay can be attributed to the standardization of the assay, reagents, and commercial availability. However, test statistics, per the package insert of commercially available assays, are compared against other ELISA assays and not culture. In the peer-reviewed literature, the cELISA, with a cut-off of 1:10 has a sensitivity of 100% and a specificity of 99.7% [120,121].

## Conclusions

Brucellosis is an ancient disease that still plagues the world, particularly developing nations. While it is the most widespread zoonosis and most common laboratory-acquired infection, there are still knowledge gaps and a need for better diagnostics and vaccines to make inroads towards control and eradication. However, over the past two decades, improvements have been made to better understand the various aspects of human and animal brucellosis. Meanwhile, large numbers of wildlife and livestock, especially in the developing world, are naturally infected with this potential bioterrorism agent. Risk factors have been clearly delineated for brucellosis in human populations, but many developing and war-torn regions lack infrastructure and funding to implement strategies to reduce these risk factors. Therefore, in the U.S., it is beneficial to society to tackle this disease at home and abroad, which will most likely increase expected benefits of control strategies. Immense challenges that remain in controlling and eradicating brucellosis are: (1) to develop and validate novel diagnostics to replace culture, ideally as an ante-mortem assay; (2) to develop efficacious vaccines that provide better protection to animal populations and are differentiation of infected from vaccinated animals (DIVA) compliant; and (3) to address the disease in the natural animal reservoirs and dedicate resources to brucellosis management in animals to reduce the incidence in human populations, effectively applying a One Health framework. Ultimately, with a disease this challenging, all stakeholders must be working together instead of against each other. This disease will not be controlled or eradicated without meaningful collaboration between, local, state, federal, private, and public partnerships.

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# Optimization of *Brucella abortus* Protocols for Downstream Molecular Applications

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**ABSTRACT** We compared the performances of various DNA extraction kits for their ability to recover *Brucella abortus* strain 19 inoculated into *Brucella*-free bovine tissues. Tissues were homogenized in a FastPrep bead homogenizer and extracted in triplicate by using one of five kits (Qiagen DNeasy, GE Illustra, Omega Bio-tek E.Z.N.A., Quanta Extracta, and IBI Science DNA Tissue kit). Whole blood was also taken from animals prior to chemical euthanasia, aliquoted, and then fractioned into buffy coat, red blood cells, and plasma. DNA was extracted from whole blood, buffy coat, and plasma by using four kits (Qiagen DNeasy, Omega Bio-tek E.Z.N.A., IBI Science DNA Blood kit, and 5PRIME PerfectPure). Previously reported primers targeting strain 19 were used to amplify extracted DNA and identify the optimal extraction kit. Real-time PCR was performed, and kits were compared for statistical differences by using quantification cycles as an outcome measure. Omega Bio-tek E.Z.N.A. was superior ($P < 0.0068$) in its lower quantification cycle values across all tissue kits. The IBI Science DNA Blood kit was superior to Qiagen DNeasy, 5PRIME PerfectPure, and Quanta Extracta ($P < 0.0001$, $P = 0.0004$, and $P = 0.0013$, respectively) but was not different from Omega Bio-tek E.Z.N.A. ($P = 1.0$). In summary, the optimal extraction kit for *B. abortus* strain 19 for tissues is Omega Bio-tek E.Z.N.A., and that for blood and its fractions is the IBI Science Mini Genomic DNA kit. Eluted DNA was also concentrated by using the Zymo Research DNA Clean & Concentrator-25 kit. Concentrated eluted DNA with the target was superior ($P = <0.0001$) to unconcentrated eluted DNA.

**KEYWORDS** brucellosis, DNA concentration, DNA extraction, epidemiology, PCR, molecular methods

While primer and/or probe identification for specific genomic targets in PCR assays is important, the processing of samples, particularly whole tissues, from suspect animals for downstream molecular diagnostics is equally important. The peer-reviewed literature contains multiple studies assessing extractions from "difficult" samples, such as feces and soil, but no data comparing various extraction techniques utilizing commercial kits for Gram-negative bacteria in tissue matrices have been reported (1–3). Our model organism is *Brucella abortus*, a Gram-negative, nonmotile, facultative, intracellular coccobacillus that is the etiological agent of brucellosis (4). Brucellae are organisms that are known to invade host tissue and reside intracellularly in low numbers (5, 6). Recent *Brucella* reports have focused on DNA extraction from whole blood, serum, and milk (6, 7). Notably, studies detailing whole-blood and serum extractions are based on human populations. Efficient and relatively inhibitor-free extraction of DNA is critical for use in downstream PCR assays.

Most PCR applications for brucellosis focus on testing postculture isolates from suspected tissues and not directly from tissues of infected animals in the field (8, 9). Most applicably, a study was conducted to determine optimal DNA extraction kits for

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human serum samples that were spiked with *Brucella melitensis* vaccine strain Rev 1 (6). However, those spiked human serum samples contained a different, albeit related, organism. Another report details procedures for DNA extraction from laboratory mice challenged with *Francisella tularensis* (10). That experiment was done with a challenge dose of 100 CFU, where challenged animals were allowed to develop clinical signs prior to euthanasia, sampling, and subsequent DNA extraction. Unfortunately, data from laboratory challenge studies are not analogous to those for clinical specimens typically received in diagnostic laboratories. There is no overlap in kits evaluated in the previous studies and kits evaluated in this study.

According to the World Health Organization, brucellosis is the most widespread zoonosis and is classified as one of the seven most neglected diseases worldwide (11, 12). In the United States, bovine brucellosis, predominantly caused by *B. abortus*, is the disease of concern due to implications for public health and national and international trade. In the United States, cattle can be infected with *B. abortus* and *Brucella suis*. There are no reservoirs of *Brucella melitensis* (13, 14). Given its proclivity as an intracellular pathogen, *B. abortus* is well documented for its evasion of the host immune system (15). Bacterial cells are voluntarily sequestered in regional lymph nodes and modulate host immune factors to decrease immune responses (16). Moreover, in infections not temporally concentrated around parturition, *B. abortus* bacteria persist in low numbers in lymph nodes and lymphoid tissues (17). Thus, bacteriological isolation of this Gram-negative, intracellular pathogen for diagnostics is hampered by this low bacterial burden.

Diagnostic testing for animal brucellosis relies on the "gold standard" of bacteriological culture. Culture is typically carried out on animals that have tested positive upon antemortem assays (i.e., serology). Presumptively positive animals are culled from their respective groups and subjected to necropsy or sampled at slaughter facilities. However, while culture is very specific, it has a low sensitivity. *B. abortus* can be cultured from only 30 to 50% of seropositive animals, leaving the true status of 50 to 70% of seropositive animals unknown (13). It is unclear whether (i) these 50 to 70% of animals are animals that have cleared the infection and have a long-lasting antibody titer, (ii) these 50 to 70% of animals have been infected with one of several serological cross-reacting organisms, or (iii) culture is unable to detect these low-copy-number organisms in tissues. Therefore, there is a need for diagnostics to move toward more sensitive methods such as PCR. However, preparation of samples for downstream molecular applications is heavily dependent on sample processing and the ability to obtain a target-rich, relatively inhibitor-free template.

Therefore, our objectives were to (i) identify the optimal commercial DNA extraction method for *B. abortus* and (ii) show DNA concentration methods that increase the quantity of available target DNA for downstream PCR amplification.

## MATERIALS AND METHODS

**Bacterial strains.** *Brucella abortus* vaccine strain 19 (S19) was used in this study (18). This strain was obtained from the Wyoming Game and Fish Department Wildlife Disease Laboratory (Laramie, WY, USA). Strain 19 was grown on Colombia blood agar plates (Hardy Diagnostics, Santa Maria, CA, USA) at 37°C with 10% $CO_2$ for 5 days. Colonies were aseptically collected from the plate and diluted in 4.8 ml of nuclease-free water.

**Blood preparation.** Five 10-ml Vacutainers containing EDTA (Becton Dickinson, Franklin Lakes, NJ, USA) were filled with 7 ml of venous blood (after sedation and before chemical euthanasia, via jugular venipuncture). Blood was spiked with 1 ml of an S19 suspension at $1.87 \times 10^7$ CFU/ml and incubated for 24 h at 39°C. Afer incubation, 400 $\mu$l of whole blood was aliquoted and used for DNA extraction. The remaining blood in the Vacutainers was then centrifuged at 3,000 rpm at 20°C for 15 min in a Beckman Coulter Allegra 6R centrifuge (Beckman Coulter, Brea, CA, USA). Plasma, buffy coat, and red blood cells (RBCs) were aliquoted following centrifugation, and 400-$\mu$l aliquots each were subsequently taken in triplicate for extraction.

**Tissue preparation.** Spleen, cervix, uterus, placentome, and supramammary, prescapular, internal iliac, and medial retropharyngeal lymph nodes from cattle (*Bos taurus*) were aseptically acquired at the Wyoming State Veterinary Laboratory from diagnostic cases originating outside the region where brucellosis is endemic. Lymph nodes were left intact, while other tissues were taken in 100-g samples. Tissues were inoculated with S19 ($1.87 \times 10^7$ CFU/ml) by using a 22-gauge needle with a 3-ml syringe. Ten 100-$\mu$l injections of the inoculum were made in different locations of the intact tissue, for a total

inoculation volume of 1 ml. Tissues were incubated in a humidified incubator at 39°C for 24 h prior to homogenization. Tissues were incubated for 24 h to allow the bacterial cells to infect the tissues in an intracellular manner, analogous to natural *Brucella* infection. In a biosafety cabinet, three ~1-g tissue pieces were aseptically collected and placed into 2.0-ml FastPrep tubes (MP Biomedicals, Santa Ana, CA, USA). Included in the tube were 0.10 g of 0.1-mm zirconia-silica beads, 0.28 g of 0.5-mm zirconia-silica beads, and 0.30 g of 1.0-mm zirconia-silica beads (Biospec Products, Bartlesville, OK, USA) in addition to 250 μl of 1× phosphate-buffered saline (pH 7.4). Tubes were placed into the Thermo Savant FastPrep FP120 instrument and run for two 30-s intervals at a speed setting of 4.5. Tubes were then centrifuged in an Eppendorf 5415D microcentrifuge (Eppendorf, Hamburg, Germany) at 12,000 × *g* for 3 min. After centrifugation, 400 μl of the supernatant from the homogenate was placed into a sterile 1.5-ml microcentrifuge tube and extracted with one of the commercial DNA extraction kits. Tissues were processed in triplicate for each kit. All animal work was approved by the University of Wyoming Institutional Animal Care and Use Committee (protocol no. 20140424BS00094-02), and all laboratory work was approved by the University of Wyoming Institutional Biosafety Committee (registration no. 20140630-60).

**DNA extraction kits.** Kits were differentiated based on suggested matrices (tissue or blood). Tissue kits included the Qiagen DNeasy (catalog no. 69506; Qiagen, Hilden, Germany), GE Illustra (catalog no. 28904275; GE, Boston, MA, USA), Omega Bio-tek E.Z.N.A. Tissue (catalog no. D3396-02; Omega Bio-tek, Norcross, GA, USA), Quanta Extracta DNA Prep for PCR (catalog no. 95091-250; Quanta Biosciences, Beverly, MA, USA), and IBI Science Mini Genomic DNA Tissue (catalog no. IB47222; IBI Science, Peosta, IA, USA) kits. Blood kits included the Qiagen DNeasy kit (catalog no. 69506; Qiagen, Hilden, Germany) for whole blood, plasma, buffy coat, and RBCs; the 5PRIME GmbH PerfectPure DNA kit (catalog no. 2302100; 5PRIME GmbH, Hilden, Germany) for whole blood, plasma, buffy coat, and RBCs; Quanta Extracta DNA Prep for PCR (catalog no. 95091-250; Quanta Biosciences, Beverly, MA, USA) for plasma and buffy coat; the IBI Science Mini Genomic DNA Blood kit (catalog no. IB47202; IBI Science, Peosta, IA, USA) for whole blood, plasma, buffy coat, and RBCs; and the Omega Bio-tek E.Z.N.A. Blood DNA minikit (catalog no. D3392-02; Omega Bio-tek, Norcross, GA, USA) for whole blood, plasma, buffy coat, and RBCs. All blood and tissue extraction kits, except for Quanta Extracta, were based on silica spin column technology; Quanta Extracta DNA Prep for PCR is an enzyme digestion extraction kit. Quanta Extracta was indicated for use with buffy coat and plasma samples only. Blood samples, in addition to tissue samples, were run in triplicate. All sets of extractions were run with an extraction control consisting of kit reagents with no biological sample. This served to indicate either kit or environmental contamination. The manufacturers' protocols were followed for each kit, and eluted DNA was stored at −20°C for further analysis.

**Quantification of DNA concentration and purity.** Each triplicate of eluted DNA from the respective kits was assessed for concentration ($A_{260}$; bichromatic absorbance correction of 320 nm) and purity (by measure of the $A_{260}/A_{280}$ ratio) on a NanoDrop 2000C instrument (Thermo Fisher Science, Waltham, MA, USA). The NanoDrop 2000C instrument was rezeroed against the elution buffer after triplicates for each kit were analyzed. Interpretation of DNA purity was based on an optimal $A_{260}/A_{280}$ ratio of 1.8 (19).

**Real-time PCR amplification.** Since the samples were spiked bovine tissues and blood, total S19 DNA was not quantifiable by the NanoDrop instrument, as host genomic DNA was also purified. Therefore, S19-specific quantification was required to elucidate optimal extraction kits. Extracted DNA from S19 was amplified by using previously reported primers targeting the erythritol catabolism (*eryC*) gene (20, 21). The *eryC* gene contains a 702-bp deletion in S19 and produces a 361-bp amplicon specific for S19. Primers were ordered from Integrated DNA Technologies (Coralville, IA, USA) and were forward primer 5′-TTGGCGGCAAGTCCGTCGGT-3′ and reverse primer 5′-CCCAGAAGCGAGACGAAACG-3′. A Bio-Rad CFX 96 Touch quantitative PCR (qPCR) thermocycler (Bio-Rad, Hercules, CA, USA) was used to amplify target DNA under the following conditions: an initial denaturation step at 98°C for 5 min, a denaturation step at 95°C for 15 s, an annealing step at 60°C for 15 s, and an extension step at 60°C for 45 s for 40 cycles. The reaction mixture was composed of 1 μl of 20 μM each forward and reverse primer (final concentration of 1 μM each), 2× (10 μl) Bio-Rad iTaq Universal SYBR green mix (Bio-Rad, Hercules, CA, USA), 1 μl of the DNA template, and 7 μl of nuclease-free water for a total reaction mixture of 20 μl. Extraction controls (absence of the biological homogenate) were run with the extraction kits to ensure that the kit components were not contaminated. No-template controls using nuclease-free water as the template were used in the PCR to ensure the absence of environmental or PCR reagent contaminants. DNA extracted from S19 colony isolates was used as a positive control. qPCR thresholds were automatically determined by using Bio-Rad CFX Manager software (version 3.1), utilizing a single threshold mode.

Melting curve analysis was performed after amplification. The hold time prior to melting curve analysis was 95°C for 5 s, followed by 65°C for 5 s, with an increase to 95°C in 0.5°C increments. SYBR green fluorescence curves were analyzed with Bio-Rad CFX Manager software (version 3.1). The melt peak was confirmed based on the melt peak of the positive control, S19.

**DNA concentration/enrichment.** Strain 19 was grown on Colombia blood agar plates (Hardy Diagnostics, Santa Maria, CA, USA) at 37°C with 10% $CO_2$ for 5 days. Colonies were aseptically collected from the plate and diluted in 4.8 ml of nuclease-free water. The strain 19 culture suspension aliquot was vortexed, and 400 μl was pipetted into 1.5-ml Eppendorf microcentrifuge tubes (Eppendorf, Hamburg, Germany). This suspension was extracted with the Omega Bio-tek E.Z.N.A. kit with a final elution volume of 400 μl. Eluted DNA from extraction kits was purified and concentrated by using the Zymo Research DNA Clean & Concentrator-25 kit (Zymo Research, Irvine, CA, USA). Two hundred fifty microliters of the eluted DNA was concentrated to 25 μl. All sets of eluted DNA were run with a concentration control, to indicate either kit or environmental contamination. The manufacturers' protocols were followed, and concentrated DNA was stored at −20°C for further analysis.

**TABLE 1** DNA concentrations, DNA purities, and 95% confidence intervals for blood, plasma, buffy coat, red blood cells, and tissues[a]

| Sample type | Kit | DNA concn (ng/$\mu$l) (95% CI) | DNA purity ($A_{260}/A_{280}$ ratio) (95% CI) |
|---|---|---|---|
| Whole blood | Qiagen DNeasy | 8.5 (8.2–8.8) | 1.92 (1.78–2.06) |
| | 5PRIME PerfectPure | 54.23 (38.2–70.3) | 1.9 (1.89–1.90) |
| | IBI Mini Genomic DNA Blood | 8.27 (7.3–9.2) | 1.79 (1.50–2.08) |
| | Omega Bio-tek E.Z.N.A. | 15.07 (9.1–21.1) | 1.79 (1.59–1.99) |
| Plasma | Qiagen DNeasy | 4.73 (4.3–5.2) | 1.96 (1.68–2.24) |
| | 5PRIME PerfectPure | 2.466 (2.2–2.7) | 1.6133 (0.71–2.51) |
| | Quanta Extracta | 136.33 (17.4–255.3) | 0.66 (0.63–0.69) |
| | IBI Mini Genomic DNA Blood | 3.533 (1.8–5.3) | 1.6733 (0.11–2.26) |
| | Omega Bio-tek E.Z.N.A. | 6.36 (6.2–6.6) | 1.6133 (1.18–2.05) |
| Buffy coat | Qiagen DNeasy | 50.63 (26.7–74.5) | 1.74 (1.66–1.82) |
| | 5PRIME PerfectPure | 99.53 (65.2–133.9) | 1.35 (0.97–1.73) |
| | Quanta Extracta | 295 (270.3–319.7) | 0.97 (0.96–0.98) |
| | IBI Mini Genomic DNA Blood | 35.1 (32.6–37.6) | 1.79 (1.38–2.20) |
| | Omega Bio-tek E.Z.N.A. | 33.6 (24.0–43.2) | 1.73 (1.65–1.81) |
| Red blood cells | Qiagen DNeasy | 12.06 (8.7–15.4) | 1.946 (1.76–2.13) |
| | 5PRIME PerfectPure | 6.366 (6.1–6.7) | 2.1266 (2.05–2.20) |
| | IBI Mini Genomic DNA Blood | 11.8 (10.6–13.0) | 1.6733 (1.34–2.00) |
| | Omega Bio-tek E.Z.N.A. | 15.76 (14.6–16.9) | 1.936 (1.89–1.98) |
| Tissues (collapsed) | Qiagen DNeasy | 103.38 (83.7–123.1) | 1.983 (1.96–2.05) |
| | Quanta Extracta | 1797.5 (1797.4–1797.5) | 1.566 (1.55–1.59) |
| | IBI Mini Genomic DNA Tissue | 279.37 (263.7–295.1) | 1.894 (1.88–1.92) |
| | Omega Bio-tek E.Z.N.A. | 308.77 (253.3–364.2) | 1.882 (1.87–1.91) |
| | GE Illustra | 315.22 (308.9–321.6) | 1.91 (1.79–2.17) |

[a]CI, confidence interval.

**Statistical analysis.** Extraction kits were compared by quantification cycle ($C_q$) values, with lower $C_q$ values indicating more-efficient amplification, which, in this design, we interpreted as an effect of the extraction method. Mean values were calculated based on triplicate runs after confirmation of amplicon size by melting curve analysis. All statistical tests were performed by using JMP Pro version 12.0.1 (SAS Institute, Cary, NC, USA). Statistically significant differences between tissue values were determined by a Kruskal-Wallis test at an alpha value of 0.05. For blood, Kruskal-Wallis statistics included a blocking factor of replicate number. The differences between unconcentrated and concentrated DNAs by the Zymo Research DNA Clean & Concentrator-25 kit were compared by a Wilcoxon signed-rank test. Statistical significance was determined as a $P$ value of $<0.05$.

## RESULTS

**Performance of blood DNA extraction kits.** The DNA concentrations ($A_{260}$), DNA purities ($A_{260}/A_{280}$ ratios), and 95% confidence intervals for blood kits are presented in Table 1. For whole blood, 5PRIME PerfectPure had the highest concentration of DNA (54.23 ng/$\mu$l), with an acceptable purity value of 1.90. In the plasma fraction of spiked samples, Quanta Extracta had a high DNA concentration (136.33 ng/$\mu$l) but poor purity ($A_{260}/A_{280}$ ratio of 0.66), indicating protein contamination. This trend was also seen for the buffy coat sample, with Quanta Extracta having the highest DNA concentration (295 ng/$\mu$l) but poor purity ($A_{260}/A_{280}$ ratio of 0.97). The second best commercial kit for buffy coat, when prioritizing DNA purity, was the IBI Science Mini Genomic DNA Blood kit, which had a purity value of 1.79 and a DNA concentration of 35.1 ng/$\mu$l. For red blood cells, Qiagen DNeasy appeared to be optimal, with the second highest DNA concentration (12.06 ng/$\mu$l) and a purity value of 1.946.

Real-time PCR was performed by using S19-specific primers to elucidate which kit yielded the lowest $C_q$ values in a SYBR green real-time PCR format. These data are included in Table 2. For buffy coat, the sample that contains the highest concentrations of target phagocytes of *Brucella*, the best-performing kit based on $C_q$ values was the IBI Science Mini Genomic DNA Blood kit ($C_q$ value of 27.72), closely followed by 5PRIME PerfectPure ($C_q$ value of 27.73). For whole blood, the Omega Bio-tek E.Z.N.A. kit had the lowest average $C_q$ (26.16), followed by the IBI Science Mini Genomic DNA Blood kit ($C_q$ of 27.41). For plasma, Omega Bio-tek E.Z.N.A. was superior ($C_q$ of 26.6), followed by the IBI Science Mini Genomic DNA Blood kit ($C_q$ of 27.04). For red blood cells, the IBI Science Mini Genomic DNA Blood kit was optimal ($C_q$ of 27.24). All extraction controls were negative by PCR ($C_q$ of $>40$).

**TABLE 2** PCR assay with $C_q$ values of kits based on various matrices and 95% confidence intervals

| Sample type | Kit | $C_q$ value | 95% CI |
|---|---|---|---|
| Whole blood | Qiagen DNeasy | 30.36 | 29.74–30.99 |
| | 5PRIME PerfectPure | 28.45 | 28.15–28.76 |
| | IBI Mini Genomic DNA Blood | 27.41 | 26.93–27.89 |
| | Omega Bio-tek E.Z.N.A. | 26.16 | 24.46–27.86 |
| Plasma | Qiagen DNeasy | 28.72 | 27.87–29.57 |
| | 5PRIME PerfectPure | 29.7 | 29.40–30.00 |
| | Quanta Extracta | 28.02 | 25.79–30.24 |
| | IBI Mini Genomic DNA Blood | 27.04 | 26.74–27.34 |
| | Omega Bio-tek E.Z.N.A. | 26.6 | 26.06–27.14 |
| Buffy coat | Qiagen DNeasy | 28.39 | 27.56–29.21 |
| | 5PRIME PerfectPure | 27.73 | 26.65–28.82 |
| | Quanta Extracta | 29.98 | 29.33–30.64 |
| | IBI Mini Genomic DNA Blood | 27.72 | 27.26–28.18 |
| | Omega Bio-tek E.Z.N.A. | 28.19 | 26.92–29.45 |
| Red blood cells | Qiagen DNeasy | 30.81 | 30.28–31.34 |
| | 5PRIME PerfectPure | 28.48 | 27.85–29.10 |
| | IBI Mini Genomic DNA Blood | 27.24 | 26.91–27.57 |
| | Omega Bio-tek E.Z.N.A. | 31.13 | 30.31–31.95 |
| Tissues (collapsed) | Qiagen DNeasy | 26.67 | 25.88–27.46 |
| | Quanta Extracta | 30.07 | 28.61–31.54 |
| | IBI Mini Genomic DNA Tissue | 25.62 | 24.96–26.29 |
| | Omega Bio-tek E.Z.N.A. | 23.94 | 23.00–24.88 |
| | GE Illustra | 29.59 | 28.73–30.44 |

There were no statistical differences in the fractions of blood. Therefore, blood and its fractions were concatenated, and a Kruskal-Wallis test was run with the blocking factor of individual replicate. For the concatenated blood, the IBI Science Mini Genomic DNA Blood kit was superior to all other kits ($P = 0.0013$), except for the Omega Bio-tek E.Z.N.A. kit, which showed no difference ($P = 1.0$).

**Performance of tissue DNA extraction kits.** The DNA concentrations ($A_{260}$), DNA purities ($A_{260}/A_{280}$ ratios), and 95% confidence intervals for tissue kits are presented in Table 1. Quanta Extracta appeared to be inferior when evaluating DNA purity. However, the Omega Bio-tek E.Z.N.A., IBI Science Mini Genomic DNA Tissue, Qiagen DNeasy, and GE Illustra kits were similar within individual tissues ($P = 0.83$). Therefore, to increase power, all tissues were collapsed and evaluated as a whole. Quanta Extracta had the highest average DNA concentration (1,797.5 ng/$\mu$l) but the lowest purity ($A_{260}/A_{280}$ ratio of 1.566). GE Illustra had the second highest DNA concentration (315.22 ng/$\mu$l), with a purity of 1.91. This was followed by similar quantification with the Omega Bio-tek E.Z.N.A. kit, with a DNA concentration of 308.77 ng/$\mu$l and a purity of 1.882.

Real-time PCR was performed by using S19-specific primers to elucidate which kit yielded the lowest $C_q$ value in a SYBR green real-time PCR format. Tissue types were concatenated by kit. The optimal kit by $C_q$ value was the Omega Bio-tek E.Z.N.A. kit ($C_q$ of 23.94), followed by the IBI Science Mini Genomic DNA Tissue kit ($C_q$ of 25.62), Qiagen DNeasy ($C_q$ of 26.67), GE Illustra ($C_q$ of 29.59), and Quanta Extracta ($C_q$ of 30.07). All extraction controls were negative by PCR ($C_q$ of $>$40).

For concatenated tissues, a Kruskal-Wallis test without a blocking factor was run to determine the likelihood of a type I error. Statistical associations are represented in Tables 3 and 4. For concatenated tissues, the Omega Bio-tek E.Z.N.A. kit was superior to all kits against which it was tested ($P = 0.0068$).

**Performance of DNA concentration/enrichment.** Preconcentrated DNA had a mean concentration of 1.32 ng/$\mu$l with a purity of 1.66. After concentration using the Zymo Research DNA Clean & Concentrator-25 kit, the mean concentration was 13.37 ng/$\mu$l, with a purity of 1.92. Before concentration, the average $C_q$ value was 37.33,

**TABLE 3** Kit-versus-kit statistical analysis to determine the optimal extraction kit for blood[f]

| | *P* value | | | | |
|---|---|---|---|---|---|
| **Kit for blood (collapsed)** | **IBI** | **Qiagen** | **5PRIME** | **Omega** | **Quanta** |
| IBI[a] | | **<0.0001** | **0.0004** | 1 | **0.0013** |
| Qiagen[b] | | | 0.0689 | 0.0782 | 0.7506 |
| 5PRIME[c] | | | | 0.2145 | **0.0193** |
| Omega[d] | | | | | 0.0997 |
| Quanta[e] | | | | | |

[a]Directionality based on a $C_q$ value of 27.35.
[b]Directionality based on a $C_q$ value of 29.57.
[c]Directionality based on a $C_q$ value of 28.59.
[d]Directionality based on a $C_q$ value of 28.02.
[e]Directionality based on a $C_q$ value of 29.32.
[f]A Kruskal-Wallis test was conducted on concatenated blood. For blood, a blocking factor on replicate number was employed. *P* values in boldface type indicate significance at a *P* value of <0.05.

which was reduced to 32.54 after concentration using the Zymo Research kit. Results are shown in Table 5. Postconcentration $C_q$ values were lower ($P < 0.0001$) than preconcentration $C_q$ values, indicating that there was more target DNA in the template. This was consistent with the DNA concentration being higher ($P < 0.0001$) in postconcentration samples than in preconcentration samples. All concentration controls for the Zymo Research kit were negative by PCR ($C_q$ of >40).

## DISCUSSION

Due to the possibility of a low bacterial burden of brucellae, efficient capture of target genomes in various clinical samples is needed to achieve the highest possible sensitivity in diagnostics while avoiding false-negative results that can confound diagnostics. The main objectives of this study were to identify optimal commercial DNA extraction kits for use with *B. abortus* and to identify a DNA concentration method that could capture low-copy-number infections by molecular diagnostics. We quantitatively assessed DNA extraction kits based on DNA concentration ($A_{260}$), DNA purity ($A_{260}/A_{280}$), and $C_q$ values on a real-time PCR SYBR green platform. However, since commercial extraction kits indiscriminately purify both pathogen and host genomic DNAs, measurement of the crude DNA concentration is not an ideal metric for selection of the optimal extraction kit. Therefore, direct quantification of S19 target DNA utilizing $C_q$ values determined by PCR was used as the determining factor for kit selection.

The Omega Bio-tek E.Z.N.A. kit is the optimal kit, of those tested, for the extraction of DNA from spiked bovine tissue samples. The Omega Bio-tek E.Z.N.A. kit was the optimal kit for the extraction of DNA from whole blood. For buffy coat, the IBI Science Mini Genomic DNA Blood kit proved to be optimal for the extraction of high quantities of relatively pure DNA. Emphasis was placed on whole blood and buffy coat, as

**TABLE 4** Kit-versus-kit statistical analysis to determine the optimal extraction kit for tissue samples[f]

| | *P* value | | | | |
|---|---|---|---|---|---|
| **Kit for tissue (collapsed)** | **Omega** | **IBI** | **Qiagen** | **GE** | **Quanta** |
| Omega[a] | | **0.0031** | **0.0002** | **<0.0001** | **0.0068** |
| IBI[b] | | | 0.0568 | **<0.0001** | **0.0064** |
| Qiagen[c] | | | | **<0.0001** | **0.0228** |
| GE[d] | | | | | 0.4636 |
| Quanta[e] | | | | | |

[a]Directionality based on a $C_q$ value of 23.94.
[b]Directionality based on a $C_q$ value of 25.62.
[c]Directionality based on a $C_q$ value of 26.67.
[d]Directionality based on a $C_q$ value of 29.59.
[e]Directionality based on a $C_q$ value of 30.07.
[f]A Kruskal-Wallis test was conducted on concatenated tissue samples. Tissues did not have a blocking factor, as all samples were independent. *P* values in boldface type indicate significance at a *P* value of <0.05.

**TABLE 5** DNA concentration and DNA purity before concentration versus after concentration with the Zymo Research DNA Clean & Concentrator-25 kit[a]

| Parameter | Value (95% CI) | | *P* value |
|---|---|---|---|
| | Preconcentration | Postconcentration | |
| DNA concn (ng/$\mu$l) | 1.32 (1.22–1.43) | 13.37 (9.08–17.66) | <0.0001 |
| DNA purity ($A_{260}/A_{280}$ ratio) | 1.66 (1.49–1.82) | 1.92 (1.81–2.03) | <0.0001 |
| $C_q$ | 37.33 (36.67–37.98) | 32.54 (30.16–34.91) | 0.0155 |

[a]A Kruskal-Wallis test was performed on DNA concentration ($A_{260}$), DNA purity ($A_{260}/A_{280}$ ratio), and real-time PCR $C_q$ values with 95% confidence intervals preconcentration versus postconcentration. All *P* values are significant at a *P* value of <0.05.

brucellae are known for intracellular infection of phagocytes and thus would be more readily found in these samples than in plasma or red blood cells (22). Additionally, in previous studies, whole blood and buffy coat were found to be optimal clinical samples for culture and PCR for human patients with brucellosis (23). While this is not directly related to chronically infected cattle, no studies have been undertaken to assess the sensitivity of PCR using whole-blood or buffy coat samples to identify infected animals. Additionally, differences in $C_q$ values are directly applicable to veterinary diagnostic laboratory assays. Typically, a delta value of 3 for $C_q$ values roughly corresponds to a log difference of amplicon target numbers (24, 25). Therefore, for kits that have lower $C_q$ values, this would increase the sensitivity of a given PCR assay. In situations where expected target DNA could be present in low copy numbers, concentration of eluted DNA is achievable with the Zymo Research DNA Clean & Concentrator-25 kit. This kit achieved a 10× concentration of the eluted DNA; thus, 1 $\mu$l of the template postconcentration is equivalent to ~10 $\mu$l of the original elution volume.

Interestingly, Quanta Extracta consistently had the highest $A_{260}$ yet suffered from low DNA purity. This was consistent with real-time PCR results, where samples extracted by Quanta Extracta consistently had the highest $C_q$ values. This kit does not make use of a silica spin column. Therefore, there is no true purification of the sample by this methodology, explaining the protein contamination seen in purity measurements.

Previous studies found that phase separation techniques that rely on protein precipitation followed by DNA precipitation are not optimal (6, 26, 27). Additionally, it is well documented that traces of phenol can completely inactivate *Taq* polymerase, thus complicating downstream applications (28). Phase separation can also be highly dependent on the technical skills of the individuals performing the extraction. The kits evaluated in this study utilized digestion with proteolytic enzymes (proteinase K) to achieve cell lysis. In comparison to phase separation techniques, commercial kits do not utilize hazardous chemicals and can be highly adoptable to a laboratory setting. All spin column kits in our experiment utilized a silica membrane. This technique utilizes enzyme digestion to release nucleic acids from cells, followed by nonspecific nucleic acid absorption to the silica fibers within the membrane. Washes of the spin column with high-salt-concentration buffers strip away low-molecular-weight compounds and residual proteins. Extraction is completed with a low- or no-salt elution buffer, which reverses the nonspecific absorption of nucleic acids from the silica fibers and into the eluate. It was reported previously that a drawback of spin column extraction kits is the potential for cross-contamination due to the aerosolization of other samples during centrifugation steps (6, 29). In our study, we ran extraction and Zymo Research concentration controls on PCR, which allowed us to evaluate cross-contamination, either by aerosolization (centrifuge and pipette, etc.) or by contamination of kit components. All extraction and concentration controls were negative by PCR ($C_q$ of >40).

While the kits tested in this study have not been extensively compared and reported, they have been widely utilized in a variety of studies. The Omega Bio-tek E.Z.N.A. kit was used previously for sampling a multitude of biological samples (30–32). Additionally, the same is true for the IBI Science Mini Genomic DNA Blood and Tissue kits (33, 34). Much has been reported on DNA extraction techniques. Unfortunately,

many of those studies dealt with methodologies that predate commercial DNA extraction kits. More recent studies evaluating commercial DNA extraction kits have focused on fungal DNA or "difficult" samples, such as soil, feces, or paraffin-embedded tissues (1, 3, 35, 36). This is the first study to assess the optimal DNA extraction kits for use on a Gram-negative intracellular bacterium from multiple matrix types.

While the capture of target DNA is vital for downstream molecular applications, protocols for the homogenization of tissues for use in commercial DNA extraction kits are equally important. In this study, we used spiked tissue samples, 3 g of which was placed into a FastPrep homogenization tube for bead beating. However, in challenge studies, the bacterial burden within lymph nodes can be as low as 17 bacterial cells per lymph node (17). Therefore, subsampling of 3 g of tissue from a lymph node introduces the risk of missing these bacterial cells for extraction. Newer technologies such as the Omni Bead Ruptor allow the homogenization of whole lymph nodes in 50-ml conical tubes. However, one would have to consider homogenization media for the use of this platform. Certain media, such as garnet-sharp particles, can have a shearing effect on bacterial cells and potentially DNA and can result in a lower sensitivity of bacteriological culture or downstream molecular diagnostic methods (37).

In summary, these results demonstrate that the Omega Bio-tek E.Z.N.A. kit was optimal for whole blood and tissues, while the IBI Science Mini Genomic DNA Blood kit was optimal for buffy coat samples. These kits showed optimal capture of target-specific DNA across inoculated matrices. These kits are easily adoptable within most laboratories and require standard equipment found in most microbiology laboratories. These kits provide high-quality eluates that can then be concentrated by using other commercial kits such as the Zymo Research DNA Clean & Concentrator-25 kit. The most efficient DNA capture methods use commercial kits, followed by concentration of the eluted DNA, which assists in increasing the sensitivity of molecular diagnostics for these intracellular, low-copy-number infections.

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All authors contributed to the design of the study. N.H., J.M., D.B., A.S., and C.K.

contributed to data gathering, cleaning, interpretation, and validation. B.S. supervised the study and contributed to the interpretation of the data. All authors contributed to the critical discussion of results and reviewed and edited the final manuscript.

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# Detection of Multiple Pathogens in Serum Using Silica-Encapsulated Nanotags in a Surface-Enhanced Raman Scattering-Based Immunoassay

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**ABSTRACT:** A robust immunoassay based on surface-enhanced Raman scattering (SERS) has been developed to simultaneously detect trace quantities of multiple pathogenic antigens from West Nile virus, Rift Valley fever virus, and *Yersinia pestis* in fetal bovine serum. Antigens were detected by capture with silica-encapsulated nanotags and magnetic nanoparticles conjugated with polyclonal antibodies. The magnetic pull-down resulted in aggregation of the immune complexes, and the silica-encapsulated nanotags provided distinct spectra corresponding to each antigen captured. The limit of detection was ∼10 pg/mL in 20% fetal bovine serum, a significant improvement over previous studies in terms of sensitivity, level of multiplexing, and medium complexity. This highly sensitive multiplex immunoassay platform provides a promising method to detect various antigens directly in crude serum samples without the tedious process of sample preparation, which is desirable for on-site diagnostic testing and real-time disease monitoring.

**KEYWORDS:** *surface-enhanced Raman scattering, multiplex immunoassay, magnetic nanoparticle, West Nile virus, Rift Valley fever virus, Yersinia pestis*

## INTRODUCTION

The development of surface-enhanced Raman scattering (SERS) spectroscopy as a detection modality for both portable diagnostic tools and high-throughput laboratory analysis has garnered significant attention over the past two decades.[1,2] The merits of SERS include the large number of Raman compounds available as reporters for a wide range of laser excitation wavelengths,[3] the use of one single excitation wavelength for multiple species,[4−6] and, above all, the narrow Raman scattering peaks that confer fingerprint-like signature profiles for each reporter. With these unique merits enabling high-level multiplex analysis,[7−9] SERS has been successfully applied to develop complex bioassays, such as multiple DNA detection[10,11] and multiple protein detection,[12] for biomedical diagnosis[13−15] and pathogen screening.[16−18] Unlike fluorescent probes, SERS nanotags are less susceptible to photobleaching and self-quenching.[19] The near-infrared laser at 785 nm used in this study can further circumvent background fluorescence issues within biological matrices.[20] Hence, the intrinsic robustness of SERS extends the application of the multiplex SERS-based immunoassays to highly complex biological matrices, such as blood,[21] embryos,[22] and *in vivo* tumors.[23]

To improve the stability of SERS nanotags and the specificity of multiplex SERS bioassays, SERS nanotags with protective shells have been developed to prevent the leaching of Raman reporters and capture biomolecules as well as to prevent cross-talk between two different unshielded SERS nanotags, which can lead to false positives.[9] Further, these SERS nanotags shield reporter molecules from reacting with contaminants in the sample and prevent the nanotags from aggregating. Several approaches have been used to fabricate the shell-protected SERS nanotags, such as silica encapsulation and polymer coatings.[24] Silica encapsulation has become widely adopted as a result of its biocompatibility, stability, optical transparency, and ease of surface modification.[25] In two independent studies,[26,27] gold nanoparticles were first incubated with Raman reporters before interacting with silane coupling agents, followed by the formation of a silica shell via Stöber's method.[28] The thiol−polyethylene glycol (PEG) compound used to stabilize gold nanoparticles,[29] however, competed with the adsorbed Raman reporters on the gold surface. To address this issue, a layer-by-layer silica encapsulation approach was developed, in which the addition of the poly(acrylic acid) layer eliminated the competitive adsorption of the thiol reagent and, thus, improved the surface coverage of Raman reporters.[20] Overall, the protective shell on the nanotags improves the performance of SERS-based bioassays in complex milieu,[24] and the silica-encapsulated nanotags have been applied to various biomedical diagnostics in biological matrices, ranging from detecting tumor cells in whole human blood[21] to multiplexed imaging in living mice.[30]

The co-infection of multiple pathogens, such as bacterial and viral pathogens, in agriculturally important animals and crops is not unusual in the same host.[31,32] In this work, we report a robust and highly sensitive SERS-based immunoassay using magnetic capture of silica-encapsulated SERS nanotags to simultaneously detect two viral antigens and one bacterial

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antigen, West Nile virus (WNV) envelope (E) protein, Rift Valley fever virus (RVFV) nucleocapsid (N) protein, and capsular antigen fraction 1 (F1) from *Yersinia pestis*, in simulated serum samples with a portable Raman instrument. Early detection of pathogenic antigens during infection has great significance in epidemic prevention and therapeutic treatment. In the early stage of viral infection, the exogenous antigen is generally of trace amount, which makes antigen detection within serum challenging. To date, only a few studies have demonstrated simultaneous detection of two or more pathogens in serum or blood with SERS-based technology. In a recent SERS-based immunoassay study, femtogram detection limits of two antigens were achieved but required hours of preparation.[33] In another advancement, the simultaneous detection of three antigens was recently reported as the first case of using the SERS-based lateral flow assay with a Raman microscope system.[34] In comparison, our SERS-based immunoassay employed a colloidal particle system instead of the solid surface system, which improved the reaction kinetics, leading to a more efficient detection.[35] To our knowledge, this work is the first report of the use of a portable Raman instrument to quantitatively and simultaneously detect three antigens of trace quantities in simulated serum samples in under one hour with no sample preparation.

## MATERIALS AND METHODS

**Reagents, Materials, and Basic Apparatus.** Colloidal gold nanoparticles (60 nm, $2.6 \times 10^{10}$ particles/mL) were purchased from Ted Pella, Inc. (Redding, CA, U.S.A.), and $Fe_3O_4$ nanoparticles (~200 nm) were purchased from Nanostructured and Amorphous Materials, Inc. (Houston, TX, U.S.A.). Bis-*N*-succinimidyl-(pentaethylene glycol) ester linker [$BS(PEG)_{12}$], succinimidyl-[(*N*-maleimidopropionamido)-dodecaethylene glycol]) ester linker [$SM(PEG)_{12}$], ammonium hydroxide, Pierce protein A/G agarose, and the Supersignal West Pico Chemiluminescent Substrate kit were obtained from Thermo Fisher Scientific (Waltham, MA, U.S.A.). HiTrap NHS-activated HP columns were ordered from GE Healthcare (Piscataway, NJ). 3-Aminopropyltrimethoxysilane (APTMS, 97%), tetraethyl orthosilicate (TEOS, 98%), 3-mercaptopropyltriethoxysilane (MPTES), 3-aminopropyltriethoxysilane (APTES), anhydrous dimethyl sulfoxide (DMSO), laser-grade Nile blue A perchlorate (NB), Janus Green B (JG), methylene blue hydrate (MB), and all other chemicals were from Sigma-Aldrich (St. Louis, MO, U.S.A.). Chemical stock solutions were made using deionized water (18.2 MΩ $cm^{-1}$) prepared with a Millipore Mill-Q A10 purification system, which were filtered by passage through 0.2 $\mu$m Millipore filtration units before use.

**WNV E, RVFV N, and F1 Antigen/Antibody [Immunoglobulin G (IgG)] Reagents.** The recombinant $His^+$-tagged WNV E protein and rabbit anti-E polyclonal antibodies have been described previously.[36] Rabbit polyclonal antibodies raised against a recombinant $His^+$-tagged fusion of RVFV N protein lacking the transmembrane domain were prepared by Drolet et al.[37] *Y. pestis* capsular antigen F1 and antibody were provided by Dr. Gerard Andrews, Department of Veterinary Sciences, University of Wyoming. Contaminating anti-*Escherichia coli* antibodies were removed from serum by total *E. coli* protein-bound HiTrap columns, and the polyclonal antibodies against F1 was then purified from the serum by affinity chromatography with a protein A/G resin. To evaluate antibody/antigen recognition specificity, replicate Western blots containing three antigens were probed individually with anti-E IgG, anti-N IgG, and anti-F IgG, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and chemiluminescence detection.

**Synthesis of Antibody-Conjugated Magnetic Nanoparticles.** The antibody-conjugated magnetic nanoparticles were synthesized as previously reported,[18] with slight modification. First, 1 mg/mL $Fe_3O_4$ nanoparticles in anhydrous DMSO were silanized by dropwise addition of APTES to a final concentration of 5% (v/v).[38] The mixture was shaken for 15 h at 40 °C, and an external magnet was then applied to collect $Fe_3O_4$ nanoparticles, followed by several consecutive washings with DMSO to remove unbound APTES. Afterward, 2.5 mg of $BS(PEG)_{12}$ linker was added to the amine-functionalized $Fe_3O_4$ nanoparticles redispersed in 1 mL of DMSO. After 6 h of incubation at room temperature, the above nanoparticle/PEG conjugates were separated from solution by magnetic collection and washed several times with phosphate-buffered saline (PBS) at pH 7.5 to remove excess PEG linker and then resuspended in 1 mL of PBS. Subsequently, three 40 $\mu$L aliquots of the nanoparticle/PEG conjugates were reacted with 200 $\mu$L of 0.2 mg/mL anti-E, anti-N, and anti-F IgGs, followed by incubation for 6 h at room temperature with gentle shaking, producing nanoparticle/PEG/anti-E, nanoparticle/PEG/anti-N, and nanoparticle/PEG/anti-F bioconjugates (referred to hereafter as capture magnetic nanoparticles), respectively. Finally, the three bioconjugates were washed repeatedly with PBS to remove unconjugated antibody and then resuspended in 240 $\mu$L of PBS for use in antigen-targeting detection assays.

**Synthesis of Silica-Encapsulated and Antibody-Conjugated SERS Nanotags.** Silica-encapsulated SERS nanotags were synthesized by a layer-by-layer method.[20] First, 20 $\mu$L of an appropriate concentration of Raman reporters (JG, $10^{-3}$ M; NB, $8 \times 10^{-4}$ M; and MB, $2 \times 10^{-4}$ M) was added to 6 mL of colloidal Au suspensions under vigorous stirring, and the mixture was equilibrated for 30 min. Next, 6 mL of poly(acrylic acid) (PAA) adjusted to pH 7.0 by 0.1 M NaOH was added dropwise to the mixture under rapid stirring for 3 h. The PAA-encapsulated nanotags were washed 3 times to remove extra Raman reporters and polymer molecules and then resuspended in water. APTMS was then added to a final concentration of $3 \times 10^{-7}$ M, followed by a 30 min equilibration step. A silica shell was subsequently grown by a modified Stöber's method. Briefly, 17 mL of 2-propanol and 200 $\mu$L of ammonia hydroxide were added successively with gentle stirring. Afterward, 12 $\mu$L of TEOS was added in six portions over a time interval of 1 h, and then the mixture was reacted for 12 h. After the addition of TEOS, centrifugation (5000 rpm for 15 min) was applied to remove free silica nuclei. The collected silica-encapsulated nanotags labeled with JG, NB, and MB were resuspended in water and adjusted to a neutral pH.

MPTES in ethanol was added to 600 $\mu$L aliquots of the silica-encapsulated nanotags to a final concentration of $3 \times 10^{-7}$ M. The mixture was incubated for 6 h at 37 °C in a shaker. After unreacted MPTES was washed away by centrifugation, 0.25 mg of $SM(PEG)_{12}$ dispersed in DMSO was added dropwise and the mixture was incubated for 6 h at room temperature with gentle shaking. Following several washes with PBS to remove excess linker and resuspension in PBS, the PEG-linked silica-encapsulated nanotags were individually reacted with corresponding IgG-enriched antiserum (0.25 mg) for 3 h at room temperature under gentle shaking. JG-labeled silica-encapsulated nanotags were functionalized with anti-E IgG; NB-labeled silica-encapsulated nanotags were functionalized with anti-N IgG; and MB-labeled silica-encapsulated nanotags were functionalized with anti-F IgG. The antibody-functionalized silica-encapsulated nanotags, referred to hereafter as reporter nanotags, were washed several times by the centrifugation/resuspension process, and the final pellets were resuspended in PBS at a desired concentration for multiplexing.

**SERS Detection of Multiple Antigen Targets by Magnetic Capture.** Multiplex assays were conducted by mixing 45 $\mu$L of each reporter nanotag, 45 $\mu$L of 20% fetal bovine serum containing dilutions of WNV E, RVFV N, and F1 antigen, and 10 $\mu$L of each capture magnetic nanoparticle. The ratio of each reporter nanotag and its corresponding capture magnetic nanoparticle was 1.1:1. Triplicate reactions for each concentration of antigen were incubated for 1 h at room temperature. The immune complexes were then separated and concentrated from solution using a small external magnet (⌀ 20 × 10 mm). The concentrated pellets were interrogated by the laser of an Advantage NIR Raman spectrometer (60 mW, 785 nm laser), (DeltaNu, Laramie, WY, U.S.A.) fitted with a right-angle input optics accessory. Spectroscopic analysis was carried out using a custom Python code in Jupyter Notebook. Raman spectra were recorded in a



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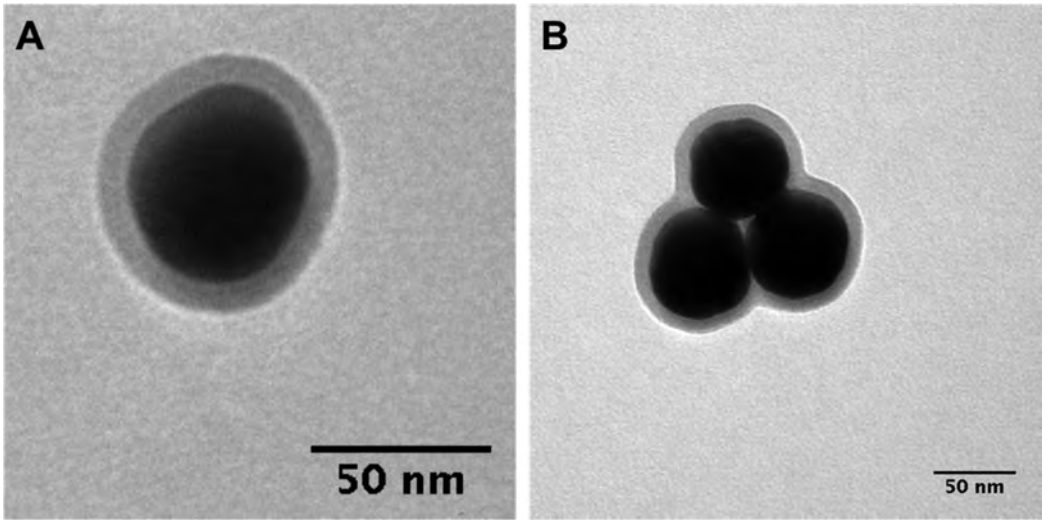


**Figure 1.** Transmission electron microscopy (TEM) images of antibody-functionalized silica-encapsulated nanotags: (A) monomer and (B) trimer.

range of 200−2000 $cm^{-1}$ with baseline off. Each recorded spectrum was an average of five independent data acquisitions (1 s integration time).

## RESULTS AND DISCUSSION

The table of contents (TOC) graphic illustrates the characteristics and mechanism of the immunoassay for target antigen detection using capture magnetic nanoparticles and reporter nanotags. Capture magnetic nanoparticles and reporter nanotags are incubated with a serum sample containing target antigens. During incubation, the polyclonal antibodies conjugated on magnetic nanoparticles and nanotags bind to different epitopes of the same antigen and form immune complexes. These immune complexes are aggregated at the interrogating laser spot by an external magnet source. For multiplex detection, immunoassays are conducted by an assembly of the three capture magnetic nanoparticles and the three corresponding reporter nanotags for WNV E, RVFV N, and F1 antigens. The specificity is based on the selection of antibody reagents that do not exhibit antigen cross recognition, the choice of Raman dyes with distinguishable peaks that do not overlap with each other, and furthermore, the use of silica-encapsulated Raman nanotags that are impervious to matrix and ionic effects, which is especially useful for detection in complex milieu, such as serum samples, where heterogeneous proteins coexist.

The thickness of the silica shell plays an important role in the performance of silica-encapsulated nanotags. A silica shell of ideal thickness should restrict massive nanoparticle aggregation, minimize its interference in laser penetration at the same time, and, more importantly, depend upon the diameter of the gold core.[29] The thickness of the silica shell synthesized on nanotags is approximately 10−15 nm (Figure 1A), which proved sufficient in blocking both the diffusion of Raman reporters away from the surface and the penetration of external moieties while still maintaining high SERS intensity. During the silica encapsulation, more than one nanotag can be encapsulated together (Figure 1B), and these clustered nanotags may enhance the Raman signal intensity better than a single encapsulated nanotag.[39]

One major difficulty in multiplex detection is the design of spectrally distinct SERS nanotags with comparable intensities. When the intensities of the three reporters vary too much, the signal of the strongest one can dominate the mixture, rendering it difficult to distinguish and quantify the signal of weaker reporters. By adjustment of the concentration of each Raman dye in the silica shell, all three reporter nanotags were able to exhibit distinguishable Raman signals with similar intensity and minimal overlap in the immunoassay for multiplex detection, which is crucial for parallel detection of multiple analytes (Figure 2).

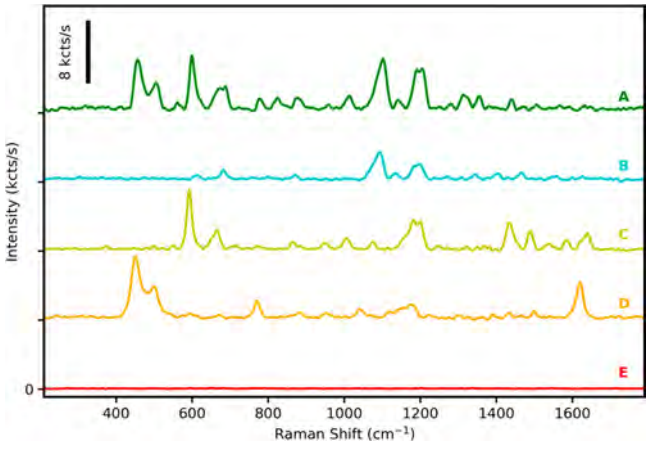


**Figure 2.** SERS immunoassays were performed in 20% fetal bovine serum and contained all three reporter nanotags and all three capture magnetic nanoparticles specific for WNV E, RVFV N, and F1 antigens: (A) all three antigens, WNV E, RVFV N, and F1, (B) WNV E antigen only, (C) RVFV N antigen only, (D) F1 antigen only, and (E) no antigen present (negative control). All immunoassays contained 10 ng/mL antigen, except the negative control. kcts/s = 1000 counts/s.

To evaluate multiplex assay performance in biological matrices, the antigens were spiked in fetal bovine serum, which was then diluted in PBS to a concentration of 20% (w/v) with an antigen concentration from 100 ng/mL to 10 pg/mL. The immunoassay yields intense spectra, as shown in Figure 2. Spectrum A is from the multiplex immunoassay detecting three antigens (10 ng/mL each) in 20% fetal bovine serum and is characterized by dominant peaks between 400 and 540 $cm^{-1}$, between 570 and 695 $cm^{-1}$, and between 1065 and 1160 $cm^{-1}$, which are a combination of three signals from MB-conjugated



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nanotags for F1 antigen detection, NB-conjugated nanotags for RVFV N antigen detection, and JG-labeled nanotags for WNV E antigen detection. Spectrum B is from an immunoassay detecting only WNV E antigen (10 ng/mL) and displays only the characteristic peaks between 1065 and 1160 $cm^{-1}$, which are corresponding to the JG-labeled SERS nanotags. Spectrum C is from an immunoassay detecting only RVFV N antigen (10 ng/mL), and the specific peaks between 570 and 695 $cm^{-1}$ are consistent with the NB-labeled SERS nanotags. Spectrum D is from an immunoassay detecting only F1 antigen (10 ng/mL), and the distinctive peaks between 400 and 540 $cm^{-1}$ match the MB-labeled SERS nanotags. Spectra B–D confirm that the silica encapsulation prevents cross-talk and increases the specificity of the multiplex immunoassay; each of these spectra shows only the characteristic peaks of one single reporter-labeled SERS nanotag. In the negative control immunoassay, all three antigens were omitted from reporter nanotags and capture magnetic nanoparticle reagents only, demonstrating negligible background peaks (spectrum E).

To determine the multiplex limit of detection for antigen capture, triplex spectra were acquired for a broad range of F1, RVFV N, and WNV E dilutions. As shown in Figure 3, a

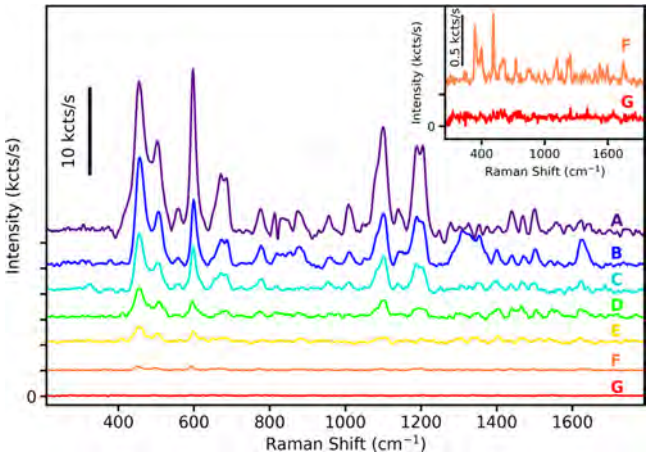


**Figure 3.** SERS spectra for triplex immunoassays of a serial dilution of the F1, RVFV N, and WNV E antigens. Stacked spectra correspond to concentrations of (A) 100 ng/mL, (B) 10 ng/mL, (C) 1 ng/mL, (D) 0.1 ng/mL, (E) 50 pg/mL, and (F) 10 pg/mL of each antigen. Spectrum G is the negative control without any antigen. (Inset) In comparison to the negative control, the characteristic peaks for a positive detection are still discernible, even at concentrations of 10 pg/mL. kcts/s = 1000 counts/s.

progressive decrease in intensity of characteristic peaks (between 400 and 540 $cm^{-1}$, between 570 and 695 $cm^{-1}$, and between 1065 and 1160 $cm^{-1}$) is observed as the concentration of the three antigens decreased from 100 ng/mL to 10 pg/mL (spectra A–F). The characteristic peaks in spectrum F, which were obtained from detecting 10 pg/mL of the three antigens, are still distinguishable compared to spectrum G, the negative control (inset of Figure 3). Therefore, we conservatively conclude that the limit of detection is a minimum of 10 pg/mL (spectrum F) in 20% fetal bovine serum.

To determine the quantitative response of SERS signal intensity to antigen concentration, multiplex spectra were background-corrected and the integration areas under the MB peaks between 400 and 540 $cm^{-1}$, NB peaks between 570 and 695 $cm^{-1}$, and JG peaks between 1065 and 1160 $cm^{-1}$ were independently plotted as a function of their respective antigen concentrations. A log–linear relationship for over 3 orders of magnitude is found from 10 pg/mL to 10 ng/mL (Figure 4),

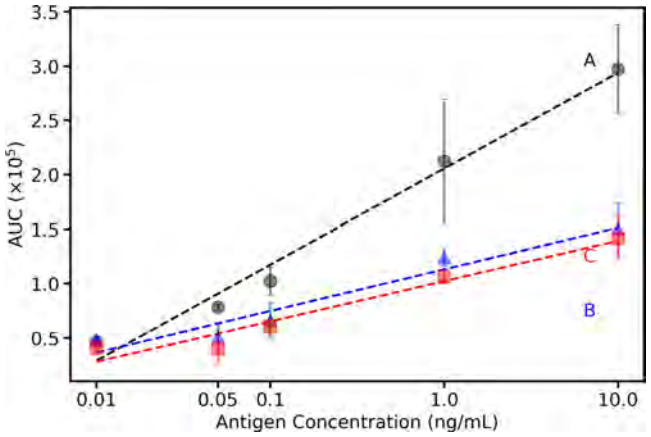


**Figure 4.** Dose–responsive curves for triplex antigen capture in 20% fetal bovine serum. Plots of the $log_{10}$ concentration of antigen versus signal intensity [area under curve (AUC)] of the characteristic peak(s) (A) between 400 and 540 $cm^{-1}$ for F1 antigen, (B) between 570 and 695 $cm^{-1}$ for RVFV N antigen, and (C) between 1065 and 1160 $cm^{-1}$ for WNV E antigen.

and linear regression analysis yields $R^2$ values of 0.9831 for F1 detection, 0.9424 for RVFV N detection, and 0.9501 for WNV E antigen. The optimal concentration of Raman reporters and the best stoichiometric ratio of reporter nanotags and capture magnetic nanoparticles are currently under investigation.

We have demonstrated that a robust, high-level multiplex immunoassay based on SERS can simultaneously detect three antigens in simulated serum samples by magnetic aggregation of the corresponding silica-encapsulated nanotag/antigen/magnetic nanoparticle immune complexes. The limit of detection for WNV E, RVFV N, and F1 antigen in 20% fetal bovine serum, 10 pg/mL, is 100-fold lower than the single antigen capture assay in 10% serum[40] and the duplex antigen detection in PBS buffer.[41] In addition to the high sensitivity, this SERS-based immunoassay has high specificity and robustness; the protective silica shell not only facilitates immobilization of the Raman reporters on the SERS nanotag surface preventing cross-talk but also blocks the unfavorable competitive adsorption of the heterogeneous proteins in serum or blood samples. Given that the sample size is of tens of microliters and demands no complicating operation, such as sample preparation, this SERS-based immunoassay can be applied to the onsite diagnostic testing and real-time disease monitoring of agriculturally relevant animals. With little modification, this modality may be adapted for the detection of a wide range of antigens in different biological matrices. Further improvements will focus on expediting assay times, improving the long-term performances of silica-encapsulated nanotags, and developing a portable Raman reader that is specially engineered for magnetic capture of nanotags.

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## Notes

The authors declare no competing financial interest.

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