



Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

A bacterial engineered glycoprotein as a novel antigen for diagnosis of bovine brucellosis



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ARTICLE INFO

Article history:

Received 14 January 2014

Received in revised form 14 March 2014

Accepted 19 April 2014

Keywords:

Bovine brucellosis

Diagnosis

Serological tests

Recombinant glycoprotein antigen

ABSTRACT

Brucellosis is a highly contagious zoonosis that affects livestock and human beings. Laboratory diagnosis of bovine brucellosis mainly relies on serological diagnosis using serum and/or milk samples. Although there are several serological tests with different diagnostic performance and capacity to differentiate vaccinated from infected animals, there is still no standardized reference antigen for the disease. Here we validate the first recombinant glycoprotein antigen, an *N*-formylperosamine *O*-polysaccharide-protein conjugate (OAg-AcrA), for diagnosis of bovine brucellosis. This antigen can be produced in homogeneous batches without the need of culturing pathogenic brucellae; all characteristics that make it appropriate for standardization. An indirect immunoassay based on the detection of anti *O*-polysaccharide IgG antibodies in bovine samples was developed coupling OAg-AcrA to magnetic beads or ELISA plates. As a proof of concept and to validate the antigen, we analyzed serum, whole blood and milk samples obtained from non-infected, experimentally infected and vaccinated animals included in a vaccination/infection trial performed in our laboratory as well as more than 1000 serum and milk samples obtained from naturally infected and S19-vaccinated animals from Argentina. Our results demonstrate that OAg-AcrA-based assays are highly accurate for diagnosis of bovine brucellosis, even in vaccinated herds, using different types of samples and in different platforms. We propose this novel recombinant glycoprotein as an antigen suitable for the development of new standard immunological tests for screening and confirmatory diagnosis of bovine brucellosis in regions or countries with brucellosis-control programs.

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A Novel Sero-Specific-Gene Dependent Multiplex PCR Enhances the Discrimination of Major *Listeria monocytogenes* Serovars

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Listeria monocytogenes is a foodborne bacterial pathogen distributed worldwide. Serotyping is extensively applied in the classification of *L. monocytogenes* and is crucial in the early stage of epidemiological tracing. Among the 13 serovars, 1/2a, 1/2b, 1/2c, and 4b are the ones most frequently isolated. Numerous PCR-based methods have been presented, however, their target genes, *lmo0737*, *ORF2110* and *ORF2819*, are prone to horizontal transfer or loss in certain strains, thus leading to incorrect serovar designation. Herein, we selected novel sero-specific genes and developed an improved multiplex PCR assay. The specificity of our assay was confirmed by the use of target and nontarget *Listeria* reference strains, as well as by the use of isolates yielding incorrect profiles in previous studies. Sensitivity tests indicated that a minimum of 5 ng of genomic DNA or approximately 3×10^6 CFU of pure culture could be detected. Many collected isolates and genomes of global isolates were used to evaluate the specificity and reproducibility of our assay. The agreement between our assay and the agglutination test was 95%, and the one between our assay and the Doumith scheme was 97%. However, our assay overcomes the drawbacks of currently used PCR-based approaches by exhibiting 100% accuracy for certain strains and clones, for instance, ST782 within the hypervirulent CC2 and ST218 that were incorrectly assigned by the Doumith scheme. In conclusion, the developed assay herein will be a powerful tool and an alternative for the classification of *L. monocytogenes* strains in foodborne outbreak investigations and surveillance programs.

Keywords: Foodborne pathogen, *Listeria monocytogenes*, serovar, sero-specific gene, multiplex PCR

Received: November 30, 2024
Revised: January 3, 2025
Accepted: January 8, 2025

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Supplementary data for this paper are available on-line only at <http://jmb.or.kr>.

pISSN 1017-7825
eISSN 1738-8872

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Introduction

Listeria monocytogenes is a Gram-positive, facultative intracellular foodborne bacterial pathogen which is the causative agent of listeriosis [1]. Individuals who are immunocompromised, elderly individuals, pregnant women, and fetuses are at high risk for listeriosis [2]. The outcomes of listeriosis can be severe, and this disease has a high mortality rate (20 to 30%), making it an important public health concern [3]. Contaminated foods, including raw staple foods and ready-to-eat processed foods, are the primary source of infection for both sporadic and epidemic listeriosis cases [4].

Due to the significance of *L. monocytogenes* characterization for epidemiological investigations, numerous subtyping methods have been reported for this important foodborne pathogen [5-9]. During the last years, WGS-based approaches have provided insights for the characterization of *L. monocytogenes* with much higher resolution and have helped elucidate epidemic events [7, 10, 11]. However, as the choice of reference genome can significantly influence subsequent analyses, the single-nucleotide polymorphism (SNP)-based WGS analysis is difficult to be standardized and may be difficult to be interpreted [7, 12]. The core genome MLST method relies on well-defined standard sets of hundreds of genes, however, there are financial and technical barriers for its application, such as the high cost of sequencing and the need for bioinformatics expertise and expensive server [13]. In particular, the lengthy turnaround time of high-quality DNA extraction and library preparation limit the application of WGS in the early stages of epidemiological investigations.

Based on serological reactions against specific somatic antigens (O-antigens; it should be noted that wall teichoic acid (WTA) represents the O-antigen of *Listeria*) and flagellar antigens (H-antigens), *L. monocytogenes* has been divided into 13 serovars [14]. Recently, another newly designated serovar, 4h, which causes severe ovine listeriosis outbreaks and is highly virulent, was reported [15]. Among these serovars, at least 95% of the isolates that originated from foods and patients were of the 1/2a, 1/2b, 1/2c and 4b serovars [16]. On the other hand, phylogenomic analysis can classify *L. monocytogenes* into four divergent lineages, and a novel hybrid sublineage



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Development of a Method for the Detection of Verotoxin-Producing *Escherichia coli* in Food

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MS 11-395: Received 26 August 2011/Accepted 25 January 2012

ABSTRACT

The growing recognition of the role of non-O157 verotoxigenic *Escherichia coli* (VTEC) in foodborne illness underscores the importance of developing methods to detect it in the food supply. We describe here the development of a protocol for the detection, isolation, and characterization of VTEC from foods, designed for the serotype-independent enrichment, detection, and isolation of VTEC, in combination with rapid characterization of VTEC O157, O26, O103, O111, and O145. This study examined the inhibitory concentration of six antimicrobial agents used either singly or in combination for the optimal enrichment of a panel of 18 different O serogroups of VTEC in modified tryptic soy broth. Considerable variability in resistance to the different antimicrobials tested was noted among different VTEC strains. The combination enabling growth of strains of all 18 different O serogroups was vancomycin (10 µg/ml) and cefsulodin (3 µg/ml). A similar combination of antimicrobials formulated in agar plates was found beneficial in the recovery of VTEC strains from enrichment broth cultures. The efficacy of these media in the recovery of selected VTEC (O26, O103, O111, O145, and O157) from ground beef and O157 VTEC from lettuce, spinach, and apple cider was demonstrated. The selective enrichment media described herein would appear suitable for incorporation in methods for the recovery and detection of a wide range of VTEC serogroups.

Verotoxin-producing *Escherichia coli* (VTEC) strains are significant food- and waterborne pathogens due to their low infectious dose (36, 37) and their potential to cause life-threatening illness (21). The terms “Shiga toxin-producing *E. coli*” and “enterohemorrhagic *E. coli*” are also used to identify these pathogens, which are associated with outbreaks and sporadic illness and are transmitted by a wide range of foods, including meat, dairy, and vegetable products (10, 30, 45). The symptoms of VTEC illness range from self-resolving diarrhea, with or without blood (hemorrhagic colitis), to life-threatening hemolytic-uremic syndrome (21). The primary virulence factors of VTEC are the verotoxins (Shiga toxins), multimeric proteins belonging to the AB5 family of toxins (18). A number of additional and putative virulence factors have been identified, which may be found in some clinical isolates (14). The most significant of these additional virulence factors is the locus of enterocyte effacement pathogenicity island (33), which has a high association with VTEC isolates from severe clinical cases and also with enteropathogenic *E. coli* (19, 20).

The first isolates of VTEC associated with severe illness were of the O157:H7 serotype in the United States (23, 32). Subsequently, regulatory and public health responses to VTEC have been focused on the serogroup O157 (serotypes O157:H7 and O157:NM). Although *E. coli*

O157 is recognized as the primary cause of VTEC illness in Canada and the United States, other serotypes of VTEC are estimated to cause 30% or more of total VTEC illness and are probably underestimated due to the absence of routine testing (1–3, 35).

Understanding the role of non-O157 VTEC in human illness and the identification of infection sources have been hampered by the shortage of validated methods for the serotype-independent detection and isolation of VTEC from foods (34). Though specific serogroups of VTEC (O26, O103, O111, O145, etc.) have been identified as more commonly associated with human illness in various regions (9, 10, 22), the serotype diversity of VTEC isolates is very high. In a review of worldwide reports of VTEC isolates, Bettelheim (5) lists 82 different O-types of VTEC for which isolation from diseased humans has been reported three or more times. That there is a high diversity of VTEC isolates is predictable since the verotoxin genes are highly mobile, being encoded by a lambda phage, the stx-phage (18). The potential for the appearance of significant VTEC pathogens possessing serotypes that were not previously associated with major outbreaks has recently been demonstrated by the appearance of a novel O104:H4 strain in Germany (13). Thus, there is a need for serotype-independent methods for the detection and isolation of VTEC to support the determination of the prevalence and risk posed by these pathogens.

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Dr. Alexander Gill



ORIGINAL ARTICLE

Development of a simple screening method for analyzing cereulide toxin in fried rice using liquid chromatography-tandem mass spectrometry

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Received: 21 August 2023 / Accepted: 8 February 2024 / Published online: 22 March 2024
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Abstract

Purpose The presence of cereulide, an emetic toxin produced by *Bacillus cereus*, in fried rice samples is critical evidence of food poisoning even in situations where *B. cereus* could not be detected. This study aims to develop a screening method for analyzing cereulide in fried rice using the QuEChERS procedure and liquid chromatography-tandem mass spectrometry (LC–MS/MS).

Methods Cereulide was identified and quantified in fried rice samples using the QuEChERS extraction method and LC–MS/MS. The accuracies of the methods were determined by analyzing fortified blank samples at two concentrations (10 and 50 µg/kg) conducted on three samples daily for five days.

Results The QuEChERS procedure removed matrix compounds from fried rice. Characteristic MS/MS spectra enabled the identification of cereulide. As the matrix effects in seven fried rice samples were within $\pm 6\%$, an external solvent calibration curve could be used for quantification. This method exhibited good accuracy ranging from 88 to 89%. The relative standard deviations for both repeatability and intra-laboratory reproducibility were $< 4\%$. These standard deviations satisfied the criteria of the Japanese validation guidelines for residues (MHLW 2010, Director Notice, Syoku-An No. 1224–1). The limit of quantification was 2 µg/kg. The applicability of this method was confirmed using the analysis of cereulide in fried rice samples incubated with emetic *Bacillus cereus*.

Conclusions The QuEChERS extraction procedure described herein showed substantial promise as a reliable screening tool for cereulide in fried rice sample.

Keywords Cereulide · Fried rice · Liquid chromatography-tandem mass spectrometry · QuEChERS · Screening

Introduction

Cereulide is an emetic toxin produced by subgroups of *Bacillus cereus*, which is widely present in the human environment and can trigger food poisoning and infectious diseases [1, 2]. At least 18 variants of cereulide are produced

by emetic *B. cereus* [3]. The cereulide toxin is dodecadepsipeptide [_D-O-Leu-_D-Ala-_L-O-Val-_L-Val]₃, with a molecular mass of 1153 Da [1, 2]. This cyclic peptide is synthesized by non-ribosomal peptide synthetases encoded by the *ces* (cereulide synthetase) gene cluster [3]. Its structural properties make it resilient against digestive enzymes, severe pH conditions, and cooking heat conditions, such as 126 °C for 90 min [1, 4]. Given its stability, cereulide remains active following oral consumption. Therefore, contamination of cooked rice dishes, such as fried rice, pilaf, and pan-fried noodles, is often the cause of outbreaks of cereulide-induced food poisoning [5–7]. Fried rice is the most typical leftover in emetic *B. cereus*-associated food poisoning in Austria and Japan [7, 8]. Since fried rice is a blend of cooked rice and other components, such as eggs, vegetables, meats, and plant

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Review

Methods used for the detection and subtyping of *Listeria monocytogenes*

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ARTICLE INFO

Article history:

Received 26 August 2011

Received in revised form 30 December 2011

Accepted 3 January 2012

Available online 9 January 2012

Keywords:

Listeria monocytogenes

Detection

Subtyping

ABSTRACT

Listeria monocytogenes is an important foodborne pathogen responsible for non-invasive and invasive diseases in the elderly, pregnant women, neonates and immunocompromised populations. This bacterium has many similarities with other non-pathogenic *Listeria* species which makes its detection from food and environmental samples challenging. Subtyping of *L. monocytogenes* strains can prove to be crucial in epidemiological investigations, source tracking contamination from food processing plants and determining evolutionary relationships between different strains. In recent years there has been a shift towards the use of molecular subtyping. This has led to the development of new subtyping techniques such as multi-locus variable number tandem repeat analysis (MLVA) and multi-locus sequence based typing (MLST). This review focuses on the available methods for *Listeria* detection including immuno-based techniques and the more recently developed molecular methods and analytical techniques such as matrix-assisted laser desorption/ionisation time-of-flight based mass spectrometry (MALDI-TOF MS). It also includes a comparison and critical analysis of the available phenotypic and genotypic subtyping techniques that have been investigated for *L. monocytogenes*.

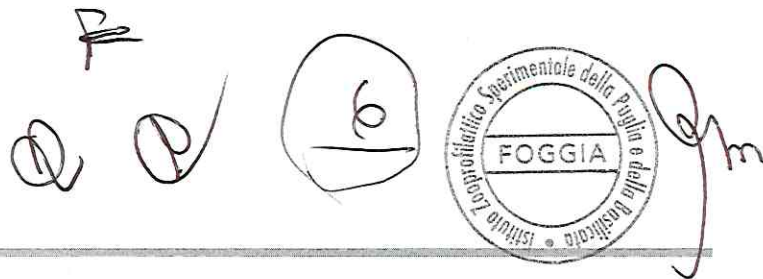
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RESEARCH ARTICLE

Rapid detection and differentiation of *Salmonella* species, *Salmonella* Typhimurium and *Salmonella* Enteritidis by multiplex quantitative PCR

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Abstract

A multiplex quantitative PCR (qPCR) was developed and evaluated for the simultaneous detection of *Salmonella* spp., *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis in various (food) matrices. Early and fast detection of these pathogens facilitates effective intervention and prevents further distribution of contaminated food products on the market. Three primer and probe sets were designed to target the *invA* gene, the STM4200 gene, and the SEN1392 gene to detect and differentiate *Salmonella* spp., *S. Typhimurium*, and *S. Enteritidis*, respectively. The multiplex qPCR targeting these three genes was optimized for efficiency and linearity. By testing 225 *Salmonella* isolates and 34 non-*Salmonella* isolates from various sources the inclusivity and exclusivity were determined. The inclusivity of the multiplex qPCR was 100% for all *Salmonella* isolates, including 72 *S. Typhimurium* isolates, and 53 *S. Enteritidis* isolates. The exclusivity for *Salmonella* spp., *S. Typhimurium*, and *S. Enteritidis* was 100%, 94.6%, and 100%, respectively. No positive results were reported for non-*Salmonella* isolates. The limit of detection (LOD) for the qPCR was determined for the matrices poultry, minced meat, egg, herbs/spices, powdered milk, fish, animal feed, boot-socks with chicken feces and chicken down. LOD values for qPCR and the conventional culture methods were similar, except for the matrix boot-socks and down, for which the LOD for the conventional culture methods performed better than the qPCR method. In conclusion, the multiplex qPCR assay developed allows for rapid screening of *Salmonella* spp., *S. Typhimurium*, and *S. Enteritidis* in various (food) matrices.

OPEN ACCESS

Citation: Heymans R, Vila A, van Heerwaarden CAM, Jansen CCC, Castelijns GAA, van der Voort M, et al. (2018) Rapid detection and differentiation of *Salmonella* species, *Salmonella* Typhimurium and *Salmonella* Enteritidis by multiplex quantitative PCR. PLoS ONE 13(10): e0206316. <https://doi.org/10.1371/journal.pone.0206316>

Editor: Anderson de Souza Sant'Ana, University of Campinas, BRAZIL

Received: July 20, 2018

Accepted: October 10, 2018

Published: October 25, 2018

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Data Availability Statement: All relevant data are within the paper.

Funding: The authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

Review

Anthrax: Transmission, Pathogenesis, Prevention and Treatment

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Abstract: *Bacillus anthracis* is a deadly pathogen that under unfavourable conditions forms highly resistant spores which enable them to survive for a long period of time. Spores of *B. anthracis* are transmitted through the contaminated soil or animal products and enter to the host through the skin, lungs or oral route and can cause cutaneous, injection, inhalation and gastrointestinal anthrax, respectively. The disease is caused by the toxin which is produced by them once they germinate within the host cell. Anthrax toxin is the major virulence factor which has the ability to kill the host cell. The role of protein kinases and phosphatases of *B. anthracis* in toxin production and other virulence related properties have also been reported. There are two vaccines, BioThrax and CYFENDUSTM, which are approved by the FDA-USA to prevent anthrax disease. Recently, anthrax toxin has also been shown to be a potential candidate for cancer therapeutics. Through present review, we aim to provide insights into sporulation, transmission and pathogenesis of *B. anthracis* as well as the current state of its prevention, treatment, vaccines and possible therapeutic uses in cancer.

Keywords: sporulation; germination; bacterial toxin; pathogenesis; anthrax vaccine



Received: 30 November 2024

Revised: 13 January 2025

Accepted: 22 January 2025

Published: 24 January 2025

Citation: Sangwan, N.; Gangwal, A.; Jain, P.; Langtso, C.; Srivastava, S.; Dhawan, U.; Baweja, R.; Singh, Y. Anthrax: Transmission, Pathogenesis, Prevention and Treatment. *Toxins* **2025**, *17*, 56. <https://doi.org/10.3390/toxins17020056>

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Key Contribution: We describe the anthrax disease and the role of toxins and other components in pathogenesis of *Bacillus anthracis*. The manuscript contains the recent developments in anthrax vaccine research and role of phosphorylation in virulence of *B. anthracis*.

1. Introduction

The life cycle of *Bacillus anthracis* is centered around two distinct phases, namely sporulation and germination, which are well-explored processes. In brief, sporulation is a multi-step process involving asymmetric septation of the vegetative bacillus resulting in the formation of two compartments, the mother cell and the forespore. Following this, the forespore remains engulfed by the mother cell and once spores mature, they are released outside the environment (Figure 1). These spores, being highly resistant, can survive and remain dormant for years until favourable conditions resume [1,2]. Once transmitted, these deadly spores can enter the host and are responsible for causing anthrax. The pathogenesis and survival of *B. anthracis* is a well-studied phenomenon. Lately, it has been reported that protein phosphorylation has a significant role in the pathogenesis of *B. anthracis* by modulating various cellular processes essential for its survival, virulence