

Development of a cyclic voltammetry method for the detection of *Clostridium novyi* in black disease

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ABSTRACT. Black disease is an acute disease of sheep and cattle. The pathogen is the obligate anaerobe, *Clostridium novyi*. Due to difficulties of anaerobic culturing in the country or disaster sites, a simple, rapid, and sensitive method is required. In this study, an electrochemical method, the cyclic voltammetry method, basing on loop-mediated isothermal amplification (LAMP), electrochemical ion bonding (positive dye, methylene blue), was introduced. DNA extracted from *C. novyi* specimens was amplified through the LAMP reaction. Then the products combined were with methylene blue, which lead to a reduction in the oxidation peak current (ipA) and the reduction peak current (ipC) of the cyclic voltammetry. The changes of ipA/ipC were real-time measured by special designed electrode, so the DNA was quantitatively detected. The results displayed that this electrochemical detection of *C. novyi* could be completed in 1-2 h with the lowest bacterial concentration of 10² colony forming units/mL, and high accuracy (96.5%), sensitivity (96%), and specificity (97%) compared to polymerase chain reation. The cyclic voltammetry method was a simple and fast method, with high sensitivity and high specificity,

and has great potential to be a usable molecular tool for fast diagnosis of Black disease.

Key words: Real-time; Cyclic voltammetry; *Clostridium novyi*; Black disease

INTRODUCTION

Black disease is an acute, highly fatal disease of sheep and cattle, which is caused by *Clostridium novyi*. *C. novyi* survives in the environment as a durable spore (Aronson and Fitz-James, 1976). Wound infection by *C. novyi* and many other *Clostridium* species causes gas gangrene (Hatheway, 1990). The onset of disease and death is usually so rapid that treatment is rarely successful or worthwhile (Parsonson, 2007). Evidence indicates that outbreaks of black disease are at least partially responsible for the massive coral mortality at the northern reef, although human activities and heat-related coral bleaching cannot be discounted as minor causes for this coral decline (Shi et al., 2012). Thus, early diagnosis and prompt treatment are important in preventing disfiguring complications.

C. novyi is a Gram-positive, endospore-forming, obligate anaerobic bacteria. It comes in 3 types, labeled A, B, and a non-pathogenic type, C. A combination of 16S rDNA and *fliC* gene sequencing of bacterial DNA isolated from spoiled meat samples revealed the unique presence of *C. novyi* type B. Although this bacterium has been implicated in clinical necrotic hepatitis in cattle, the cases described here are the first implicating *C. novyi* type B as a cause of bovine meat spoilage (Eeckhaut et al., 2012). The existing tools for diagnosis include microscopy, antigen/enzyme detection kits, and molecular tools. Microscopic diagnosis is the old method, remaining the only feasible options in many endemic countries. Anaerobic cultivation is currently used as the “gold standard”. It was commonly applied in phylogenetic analysis of the 16S RNA gene sequence of *C. butyricum* (Belokopytov et al., 2012). However, it requires relatively high-standard laboratory facilities and is a time-consuming process (Brazier et al., 2002). Badenoch et al. (2007) pointed out that anaerobic bacteria are rarely implicated in infections. In his laboratory, only 3 of a series of 432 bacterial isolates from the cornea were anaerobes. This fraction is not unusual. The real reason was likely due to lack of anaerobic culture or failure to culture due to errors in the collection, transport, detection, and other steps. Therefore, a simple, rapid, and accurate detection method of anaerobic bacteria is required.

Recently, polymerase chain reaction (PCR) has become the common method of DNA detection. On the basis of PCR, scientists have developed a series of technologies, such as quantitative real-time PCR (An et al., 2012), multiplex PCR (Traverso et al., 2006), chip PCR (Cooney et al., 2012), microfluidic PCR (Yue et al., 2012), and isothermal PCR (Paris et al., 2011). Loop-mediated isothermal amplification (LAMP) was invented by Dr. Notomi in 2000 (Notomi et al., 2000), and is characterized by its rapidity, high specificity, and high sensitivity. It uses the *bst* DNA polymerase in a constant temperature (65°C) and completes DNA amplification in 20-60 min. In recent years, LAMP has contributed to progress in detections of viruses (Wang et al., 2012), bacteria (Sowmya et al., 2012), parasites (Abdul-Ghani et al., 2012), food safeties (Zhang et al., 2012), and animal embryo sex identifications (Nogami et al., 2008). LAMP is relatively simple and could amplify DNA with high specificity. With the development of gene chips, more attentions has been paid to PCR or LAMP reactions on mi-

crofluidic gene chips. Due to the small volume, the electrochemical detection method is more suitable for the detection of DNA on the gene chip (Chang et al., 2005).

In this paper, we propose an electrochemical method, the cyclic voltammetry method, based on LAMP, for the detection of *C. novyi*. First, the DNA of *C. novyi* extracted from specimens was amplified through the LAMP reaction. Then, the products were combined with positive dyes such as Methylene blue (Xu et al., 2012), leading to a reduction in the oxidation peak current (ipA) and reduction peak current (ipC) of the cyclic voltammetry. The changes of ipA/ipC were real-time measured by a specially designed electrode, allowing the DNA to be quantitatively detected. Finally, the cyclic voltammetry method was compared with PCR, to test its accuracy, sensitivity, and specificity. Therefore, the goals of early diagnosis and prompt treatment of Black disease could be achieved. The scheme of the experiment is shown in Figure 1.

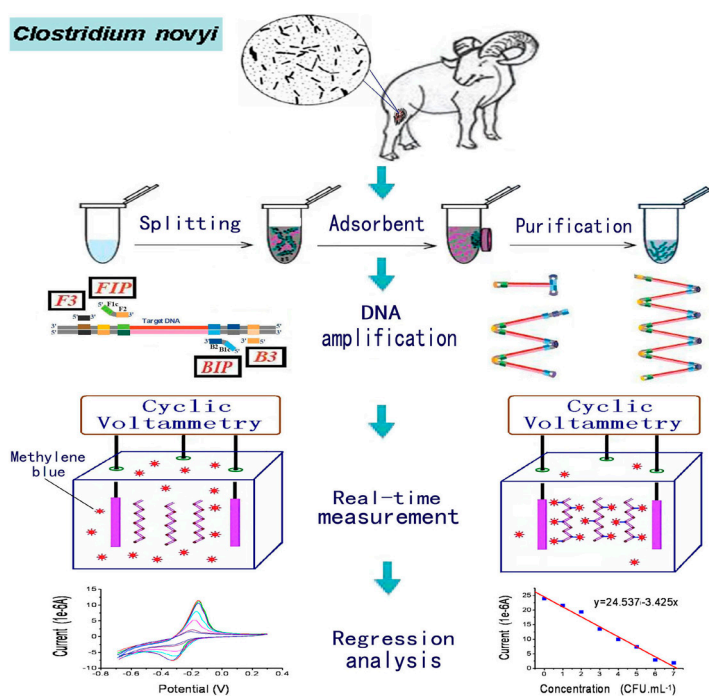


Figure 1. Scheme of *Clostridium novyi* detection by the cyclic voltammetry method.

MATERIAL AND METHODS

Reagents

The LAMP reagents were purchased from Eiken Chemical (Japan). The DNA extraction reagents were provided by Tiangeng (China). The LAMP primers were designed by PrimerExplorer 4.0 online (Eiken, Japan), and the PCR primers were designed by PrimerPremier 5.0 (Premier; Canada). The methylene blue was obtained from Sigma-Aldrich (USA) and diluted to appropriate concentration prior to use. All other reagents were analytical grade. The anaerobic mediums contained, per liter: yeast extract, 10.5 g; peptone,

12.5 g; glucose, 5 g; soluble starch, 1 g; NaCl, 5 g; sodium acetate, 3 g; and cysteine HCl, 0.6 g, were made by Pangtong (China). All solutions were prepared using Millipore-Q water (≥ 18 M Ω).

Bacterial strains and growth conditions

Three types standard strains of *C. novyi* (type A: ATCC 19402, type B: ATCC 25758, C: ATCC 27323), and 10 kinds of other bacteria standard strains which need to be differentiated: *C. perfringens* (ATCC 13124), *C. difficile* (ATCC 9689), *C. tetani* (ATCC 19406), *C. histolyticum* (ATCC 19401), *C. septicum* (ATCC 12464), *Acinetobacter baumannii* (ATCC 19606), *Enterococcus faecalis* (ATCC 14506), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), were obtained from American Type Culture Collection (ATCC; USA), grown on the corresponding medium (Pangtong; China), 24-72 h, and preserved in normal saline (NS) at -20°C until detection.

Animal model and ethical approval

The samples used in this study were obtained from sheep Black diastase models. Five sheep (average 1 year old and 30 kg) were obtained from the experimental animal center of Third Military Medical University [laboratory animal license: SYXK(YU)20070002]. The experiments were approved by the ethics committee of Xinqiao hospital. The sheep were judged to be healthy based on complete physical and microbiological examinations. Two wounds (5 cm wide and 3 cm deep) were made in back legs and injected 2 mL *C. novyi* type B (ATCC 25758) in broth medium (10^6 CFU/mL). The wounds were well bandaged after operation to create anaerobic conditions. The sheep were given normal feed for 1-5 days, and the performance of the wound was observed. The sheep infected successfully would have fever, local wound ulceration, skin blackening, gas gangrene, or be dead. Autopsy revealed hepatic necrosis and other symptoms. Blood, urine, wound exudates, liver tissue, and necrotic skin tissue were collected. One hundred specimens (20 different sites and types specimens from 5 sheep) were collected to detect *C. novyi* and preserved at -20°C.

Primer design and synthesis

The nucleotide sequences expressed specifically in *C. novyi* were retrieved from Genbank, National Center for Biotechnology Information (NCBI) as target genes. The LAMP primers were designed using PrimerExplorer 4.0 (Eiken) online. The primers were designed according to conserved regions. All primers were synthesized by Sangong (Shanghai, China). The primers were stored at -20°C and were diluted in appropriate buffer prior to use.

Optimization of the cyclic voltammetry measurement

Conventional detection (>1.0 mL) was performed using SPE glassy carbon electrode

61208110 (Metrohm, Spain). Small sample detection (<1.0 mL) was performed using platinum electrodes (designed and made by our laboratory). The platinum electrodes included a working electrode (platinum electrode), an opposing electrode (platinum electrode), and a reference electrode (Ag-AgCl electrode). The DNA amplifications were reacted in the thermobath ALB64 (Finepcr, Korea). The electrochemical detections were performed on the 910 PSTAT mini system (Dropsens, Spain). The DNA of 1.0 mL *C. novyi* type B (1×10^5 CFU/mL) was extracted by using the bacteria DNA extraction kit. The DNA amplification was carried out in a specific reaction tube containing 2 μ L template DNA, 12.5 μ L reaction reagent, 1.0 μ L bst DNA polymerase, 4 μ L primer mixture (containing 4 primers: 10 μ M F3 and B3, 40 μ M FIP, and BIP), 1 μ L 2.0 mg/L Methylene blue, and 4.5 μ L double-distilled water. The mixture in the reaction tube was incubated isothermally at 65°C for 60 min. The ipA/ipC of cyclic voltammetry was completed on the OriginPro 7.5 software (OriginLab; USA).

Specificity analysis of the method

Three types of *C. novyi* and 10 kinds of other bacteria were commonly found in infected wounds (*C. perfringens*, *C. difficile*, *C. tetani*, *C. histolyticum*, *C. septicum*, *A. baumannii*, *E. faecalis*, *E. coli*, *S. aureus*, and *P. aeruginosa*). They were cultured, counted, diluted to the same concentration (10^6 CFU/mL), DNA extracted, and *C. novyi* measured in the same way.

Sensitivity and regression analysis of the method

The *C. novyi* type B (ATCC 25758) were grown at 37°C for 24 h on Columbia blood agar plates (Pangtong; China). The counts were performed by plating 100 μ L dilutions in sterile phosphate buffer saline on plate and incubating for 24 h at 37°C. Then, the average number of CFUs were counted. *C. novyi* solution was diluted into a serial of concentrations (from 1×10^0 to 1×10^6 CFU/mL), DNA extracted, and *C. novyi* measured in the same way. The semi-logarithmic linear regression was analyzed by using the OriginPro 7.5 software.

Comparative analysis with PCR

The samples were divided into 2 parts. One part was identified by the cyclic voltammetry measurement, and the other part was amplified by PCR. The PCR assay, using 25 μ L multiplex-PCR mixtures, contained 2.0 μ L primers F and B (Table 1), 12.5 μ L 2X Fast qPCR MasterMix (Eurogentec Deutschland GmbH; Germany), and 2.5 μ L template DNA. Thermal cycling conditions comprised a uracil-n-glycosylase (UNG) step at 50°C for 2 min, hot start DNA polymerase activation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 3 s, annealing at 64°C for 30 s, and extension at 72°C for 10 s. The products were analyzed by electrophoresis on 2% agarose gels (Biowest, Spain) at 100 V for 50 min, and visualized in a Kodak Gel Logic 212 PRO Imaging System (Eastman Kodak, USA). The results were compared with difference (χ^2 test), accuracy, sensitivity, and specificity.

RESULTS

Primer design of *C. novyi*

The alpha-toxin gene (GenBank: Z48636) of *C. novyi* was selected to design the primers of *C. novyi* by exploring the GenBank database of NCBI. The specificity of primers was positively tested by using the BLAST tool of NCBI (<http://www.ncbi.nlm.nih.gov/blast>) (Table 1).

Table 1. Primer design for *Clostridium novyi* detection.

LAMP Primers (5'→3'):	
F3:	GTACTTGAAACAACCCCTAA
B3:	AAGTCTGTTAGTATTCTTGCTT
FIP:	CTTCAGGTTTAAATCCAGATGGATGGAAGTAGCTTTTATCAGC
BIP:	TTCACGAAACAAGCCATTTCTCGAAATGGCTTGTTTCGTTA
PCR Primers (5'→3'):	
F:	GTACTTGAAACAACCCCTAAA
B:	AAGTCTGTTAGTATTCTTGCTT

Optimization of the cyclic voltammetry measurement

The DNA was amplified through the LAMP reaction and the DNA amplification products were combined with positive dyes (such as methylene blue), leading to a reduction of free dye and could be real-time monitored by using the cyclic voltammetry methods (Figure 2, part A). With the passage of time, the oxidation peak current (ipA) was reduced, but in 30 min, the ipA reduced more quickly (Figure 2, part B). Therefore, we used 30-min intervals as the main monitoring points in ipA measurements of cyclic voltammetry in subsequent tests. The reduction of the ipA/ipC of the cyclic voltammetry was proportional to the amount of DNA; thus, the copies of template DNA were quantitatively detected.

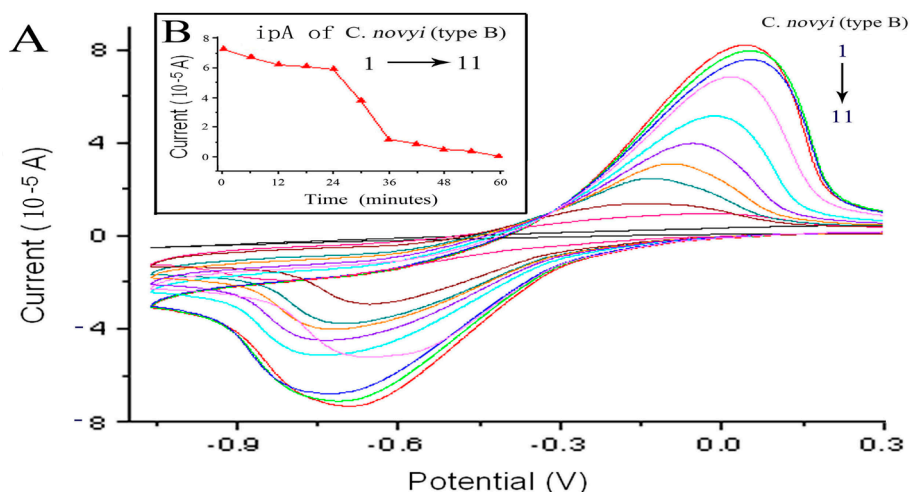


Figure 2. Optimization of cyclic voltammetry measurement. **A.** Cyclic voltammograms with the change of reaction time. **B.** ipA values of cyclic voltammetry with the change of reaction time. (Reaction time: from 1-11: 0 to 60 every 6 min.). Measured for DNA of 1.0 mL *Clostridium novyi* type B (1×10^5 CFU/mL).

Specificity analysis of the method to *C. novyi*

Figure 3 shows that the 3 types of *C. novyi* (types A, B, and C) were positively detected and the 10 other kinds of bacteria were negatively detected. This suggested a high specificity of the LAMP assay for detection of *C. novyi*. Furthermore, the quantitative determination of *C. novyi* was more easily achieved by the cyclic voltammetry analysis.

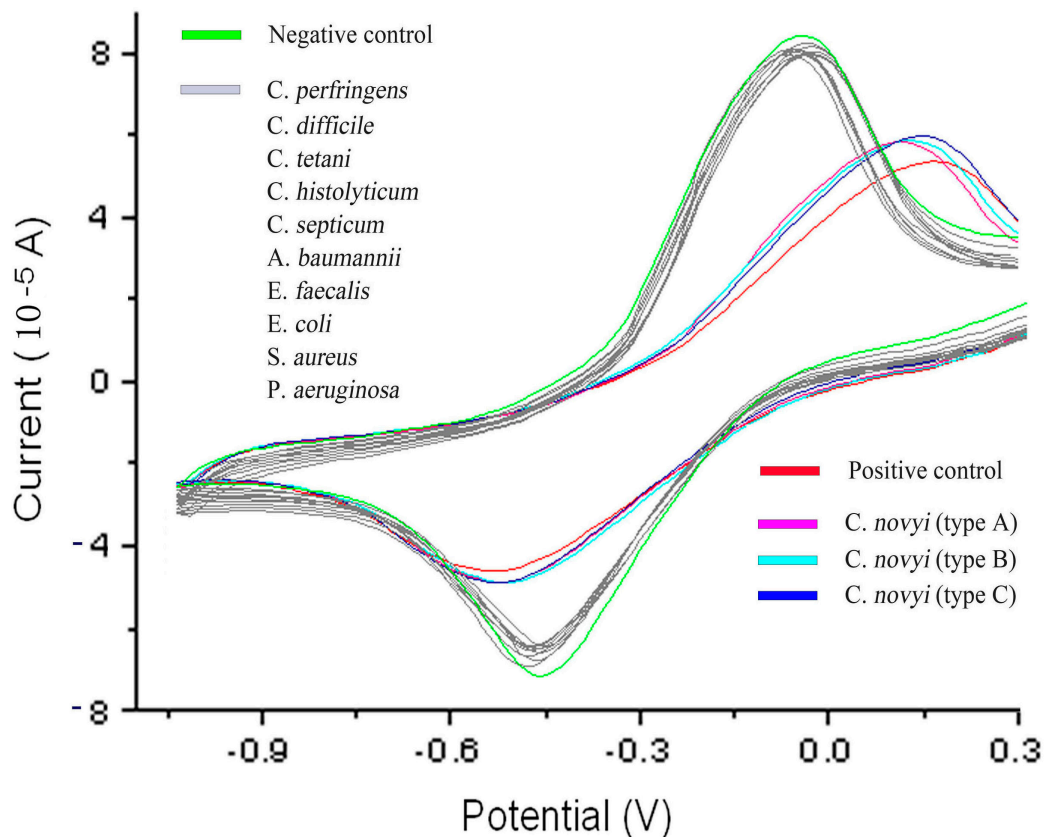


Figure 3. Specificity analysis of cyclic voltammetry measurement. Three types of *Clostridium novyi* and 10 kinds of other bacteria were measured for DNA in the same way of 1.0 mL bacteria (1×10^5 CFU/mL).

Sensitivity and regression analysis of the method

The results of cyclic voltammetry measurement showed that the detection limit of cyclic voltammetry measurement for *C. novyi* was lowest at 1×10^2 CFU/mL, indicating a high sensitivity (Figure 4, part A). A linear relationship between the *C. novyi* concentration and the ipA of cyclic voltammetry over a range of 1×10^0 to 1×10^6 CFU/mL was obtained ($y = -1.3511x + 8.1061$, $R^2 = 0.9755$) (Figure 4, part B). The quantification of the *C. novyi* copy number in the samples could be extrapolated by the cyclic voltammetry measurement.

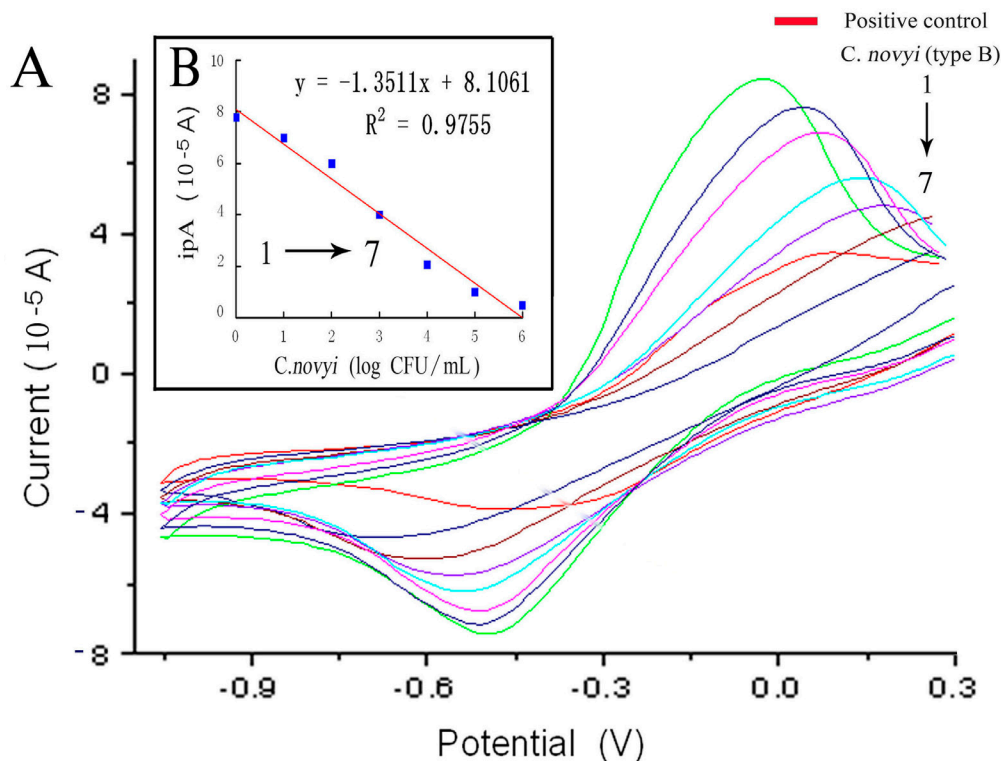


Figure 4. Sensitivity of cyclic voltammetry measurement for *Clostridium novyi* type B. A serial of concentrations (1-7: 1×10^2 to 1×10^6 CFU/mL) were measured for DNA in the same way of 1.0 mL *C. novyi* type B.

Comparative analysis with PCR

The results of comparison between the cyclic voltammetry measurement and PCR identification (Table 2) suggested that there were no significant differences between the cyclic voltammetry measurement and anaerobic culture identification ($P > 0.05$). The cyclic voltammetry measurement had high accuracy (96.5%), sensitivity (96.0%), and specificity (97.0%) compared to the standard anaerobic cultivation method.

Table 2. Comparison analysis with PCR.

Cyclic voltammetry	PCR		χ^2 test		Accuracy	Sensitivity	Specificity
	+	-	χ^2	P			
+	96	4	0.1429	0.7055*	96.5%	96.0%	97.0%
-	3	97					

*There is no statistical difference between the two methods ($P > 0.05$).

DISCUSSION

The Black disease is an acute, highly fatal disease of sheep and cattle, caused by *C. novyi* type B (Bagadi and Sewell, 1973). It may be present on skin surfaces and is a potential

source of wound infections (Plomp et al., 2007). The lethal and necrotizing toxins (primarily α toxin) damage hepatic parenchyma, thereby permitting the bacteria to multiply and produce a lethal amount of toxin (Guttenberg et al., 2011). Most of characteristic lesions are the grayish yellow, necrotic foci in the liver that often follow the migratory tracks of the young flukes. Usually, there is extensive rupture of the capillaries in the subcutaneous tissue, which causes the adjacent skin to turn black, leading to so-called Black disease. The onset of disease and death is usually so rapid that treatment is rarely successful or worthwhile. Vaccination effectively prevents Black disease, but vaccination for Black disease will not prevent death or loss of production due to liver fluke infestation. Early diagnosis and prompt treatment are very important in preventing sheep and cattle from Black disease.

C. novyi is an obligate anaerobic bacterium of the Clostridia class. It is pathogenic, causing a wide variety of diseases in humans and animals. It comes in 3 types, A, B, and a non-pathogenic type, C, that are distinguished by the range of toxins they produce. The existing tools for the diagnosis of Black disease include microscopy, antigen/enzyme detection, anaerobic cultivation, and molecular detection. Microscopy and antigen/enzyme tests remain the only feasible options for Black disease detection in many endemic countries. They can provide quantitative data and can identify species when used appropriately. Anaerobic cultivation and identification is currently used as the “gold standard” technique. However, anaerobic cultivation requires relatively high-standard laboratory facilities and is time-consuming, consequently leading to a delay beyond the optimal time window for treatment. The nucleic acid-based molecular methods are potentially good alternatives as they can accurately differentiate all species and detect at low levels. PCR-based diagnosis recently helped to identify samples that had been misdiagnosed using microscopy. The LAMP method is relatively simple and can be improved for better use in endemic countries. Compared with traditional PCR, LAMP is simpler, faster, and does not require expensive equipment. In recent years, LAMP technology has made progress in detections of viruses, bacteria, parasites, food safety, and animal embryo sex identifications. It had become a used in routine detections of the pathogenic microorganisms.

In this paper, we proposed an electrochemical detection method, cyclic voltammetry measurement, for the amplification and detection of DNA. First, the *C. novyi* Black disease toxin gene from NCBI (GenBank: Z48636) was selected as the target gene, and the primers were designed to target the relatively conserved regions of the gene identified through sequence alignments. Then, DNA extracted from specimens was amplified through the LAMP reaction. Then, the products were combined with the positive dye Methylene blue, leading to a reduction in the oxidation peak current (ipA) and a reduction in the peak current (ipC) of the cyclic voltammetry. The changes of ipA/ipC were real-time measured by a specially designed electrode. With the passage of time, the oxidation peak current (ipA) and reduction peak current (ipC) were reduced, but in 30 min, the ipA reduced more quickly; therefore, 30 min was taken as the main monitoring point in ipA measurements of cyclic voltammetry in the following tests. The results suggested that it had good specificity in detecting *C. novyi* and could accurately measure 3 types of *C. novyi*. Because of the lack understanding of gene sequence of *C. novyi*, we cannot currently distinguish the 3 types by the cyclic voltammetry measurement; however, this does not affect the diagnosis of Black disease. Combined with microscopy and antigen or enzyme tests, we could ultimately identify *C. novyi*. Furthermore, the results displayed that this electrochemical detection of *C. novyi* could be completed within 1 to 2 h with bacterial concentrations as low as 10^2 CFU/mL. The results of comparisons between the cyclic voltammetry measurement and PCR identification suggested that there were no signifi-

cant differences between the 2 methods ($P > 0.05$). The cyclic voltammetry measurement had high accuracy (96.5%), sensitivity (96.0%), and specificity (97.0%) compared to the standard PCR method.

In conclusion, the cyclic voltammetry measurement was shown to be a simple and fast method for *C. novyi* diagnosis, with high sensitivity and high specificity, and therefore has great potential to be a usable molecular tool for the fast diagnosis of Black disease.

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