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Original Article

Combined Molecular and Biochemical Identification of Alpha Toxin in Local Isolated *Clostridium novyi* from the Sheep Liver

Emadi, A[1], Pourbakhsh, S. A[2], Fathi Najafi, M[2]*, Jamshidian, M[1], Amini, K[2]

1. Department of Pathobiology, Faculty of Veterinary Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran
2. Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

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Corresponding Author: najafi99@yahoo.com

Abstract

Clostridium novyi (*C. novyi*) causes deadly Black disease in sheep and rarely in other animals. Alpha toxin (α-toxin), the most apparent pathogen of this disease, is produced by *C. novyi* type B. Economic damages of *C. novyi* include sheep mortality costs, depreciation of affected farms, and health problems with infected carcasses. The identification of *C. novyi* and isolation of its pathogens by conventional methods is a time-consuming process, necessitating a simple and rapid method for isolating and detecting pathogenic *C. novyi*. Therefore, this study aimed to molecularly identify α-toxin in local *C. novyi* isolates from the sheep livers. In this study, 75 livers suspected of Black disease were sampled. The samples of the liver were cultured under anaerobic conditions. Some of the cultured colonies were used in biochemical tests. For molecular confirmation, the DNA of isolates was extracted, and the isolates were confirmed by the polymerase chain reaction (PCR) on the liver tissue and cultured samples using specific α-toxin primers. The PCR on α-toxin produced a band in the range of 609 bp, indicating that the samples belonged to *C. novyi*. According to the results, of 75 isolates, 18 isolates were confirmed as *C. novyi*. *C. novyi* type B was isolated from the liver and confirmed by biochemical and molecular characterization. The PCR assay ensured a sensitive and specific tool for the detection of *C. novyi* in the samples.

Keywords: *Clostridium novyi*, Isolation, PCR

1. Introduction

Clostridia are a large group of spore-forming bacteria, causing pathogenicity through producing active toxins (1). *Clostridium novyi* (*C. novyi*) is an anaerobic, sporulated, Gram-positive bacillus, the flora of which exists in soil, sheep liver, and gastrointestinal tracts of some terrestrial and aquatic animals. This bacterium secrets eight primary and secondary toxins during its growth, causing a wide range of diseases in humans and animals (1, 2). Based on the type of toxin produced, *C. novyi* is divided into pathogenic types A and B, and the non-pathogenic type C (3). Alpha and beta toxins (α- and β-toxin) are among the primary *C. novyi* toxins. When this bacterium proliferates in the tissue, it produces pathogenic agents, including α-toxin, causing severe and painful localized edema, leading to necrosis and death (4, 5).

The type B bacterium causes Black disease in sheep and goats. Spores of this bacterium enter the animals' bodies through the gastrointestinal tract and penetrate the liver. Once necessary conditions, such as hypoxia and liver damage (migration of parasite larvae), were established;

moreover, latent spores are activated and proliferated, secreting α- and β-toxin (6). α-toxin is lethal and necrotic, causing morphologic changes in various types of cells, particularly endothelial cells, and failure of the skeletal structure. α-toxin with an approximate molecular weight of 200-250 kDa is enzymatically active, affecting rho receptors of mammalian cells, causing vascular bleeding and cell rounding in the culture medium (4, 7).

The detection of clostridial diseases and isolation of the pathogen by conventional methods are usually based on clinical signs, cultured colony morphology, toxin production, and immunological methods. However, these methods are time-consuming, and a simple and rapid method is required for isolating and detecting pathogenic clostridia. Therefore, toxin and 16S rRNA genes in clostridia species are often used as targets in diagnostic PCR (8-11).

Among the three *C. novyi* types, type C is not pathogenic, and NT *C. novyi* is a toxin-free strain (12). Accordingly, molecular testing based on the toxin gene is necessary for differential diagnosis.

Given the high molecular weight, long length, and instability of the third structure (approximately 6,000 codebases) of the toxin, it is difficult to diagnose the whole genome, needing high diagnostic costs. However, based on the gene bank reports, a report on the whole genome and three reports on various fragments of the α-toxin gene could be reported for common fragments to compare genomic changes (13). Due to livestock imports from other countries, lack of proper quarantine, and concerns about the diversity of isolates, the exact diagnosis of the pathogen and the infection rate in different regions of Iran and the world play a key role in preventing Black disease.

There is no molecular study in Iran on the isolation of *C. novyi* from suspicious isolates and the determination of its pathogenicity based on toxin production. The results of this study on the isolation and molecular identification of field isolates may reveal possible genotype and phenotype (pathogenicity) similarities and differences of isolates and the vaccinal strain. It is noteworthy that the produced toxin in the local isolates is considered in the cellular unit of the bacterium and gene expression. Considering the similarity of the reported toxins, there may be no genetic change in the gene and protein structure and even the toxin function in terms of enzymatic mechanism (9). However, there may be differences in toxin expression rate and speed for each isolate. Given the genomic similarities of isolates, there may be differences in toxinogenesis and pathogenicity of isolates. Accordingly, this study is necessary to identify local isolates.

2. Materials and Methods

2.1. Sample Collection

Liver samples suspected of Black disease (with necrotic zones and parasitic infection) were collected from slaughterhouses across Iran (Shiraz, Shahrekord, Ahvaz, Marand, Ilam, Mughan, and Shahriar) in six months and stored at -20˚C in a freezer until the tests. To isolate the bacterium, pieces of various liver parts were cut and crushed in a mortar and then centrifuged at 4000 g for 10 min. The supernatant was collected as extract and stored at -20˚C in a freezer until the tests. Subsequently, the supernatant was cultured in the Clostridia medium under anaerobic conditions for 24-72 h. Some of the cultured colonies were stored in a microtube at -20˚C in a freezer for subsequent tests.

2.2. Isolation of *Clostridium novyi*

A bacterial loop in the microtube was cultured on the chopped liver broth medium under anaerobic conditions for 24-48 h (HiMedi co. instructions, M606). Pure colonies were examined by common laboratory methods in terms of macroscopic properties (apparent shape of the colony), such as color, size, shape, roughness, optical properties, and in terms of microscopic properties by the gram and spore staining. To evaluate beta hemolysis by the bacterium, the plate was placed under anaerobic jar conditions, and hemolysis was analyzed after 24 h (9).

2.3. Polymerase Chain Reaction, Isolation, and Identification of Alpha-Toxin Gene

For PCR test, liver samples were processed for DNA extraction, and they were extracted by the phenol-chloroform method and that of the cultured samples by a kit

(Cinnagen, Iran). The genomic DNA was extracted by the CinnaGene Extraction Kit based on the manufacturer's protocol. In the phenol-chloroform extraction method, 500 µL of the tissue extract was centrifuged at 10000 g for 10-15 min. The resulting precipitate was dissolved in 500 µL of TE (Tris/EDTA) buffer. Ten milligrams of lysosome were dissolved in 1 mL distilled water; moreover, 10 µL lysosome and 5 µL K proteinase were added to each microtube and kept at room temperature for 10 min. To each microtube, 50 µL of 1% sodium dodecyl sulfate was added, gently mixed, and incubated at 37˚C for 20-30 min. Afterward, 500 µl phenol and 500 µl chloroform were added, and the microtube was placed in ice for 10 min. The microtubes were centrifuged at 10000 g for 10 min. The resulting precipitate was washed with 70% ethanol and dissolved in 50 µL distilled water after drying (9, 14, 15).

The quantity and quality of the extracted DNAs were evaluated by measuring their absorbance.

The PCR pair primers for the reaction and diagnosing pathogenicity based on the α-toxin were listed in table 1. For each PCR, a 20 µL reaction mixture was prepared consisting of 10 µL of the reaction mixture, 1 µL of each primer (Table 1), 2 µL of the template DNA, and 6 µL of water. Two samples were prepared for positive (CN804; *C. novyi* type B; 609 bp) and negative controls (water was added equivalently instead of DNA). The PCR was performed under the following conditions:

Initial denaturation at 94˚C for 5 min, followed by 30 cycles of denaturation at 94˚C for 30 sec, annealing at 52˚C for 30 sec, and extension at 72˚C for 1 min, with a final extension at 72˚C for 10 min. Electrophoresis was performed on 2% agarose gel, and the gel was stained with Red Safe.

Table 1. Primers used in this study

Primer name	5'→3'	bp	Position	size
F7	AAA ATT ACT GGT GAG ACA TCA GTT ATT	27	2725	609
Rc7	ACC AAC TAA TAT ACC TGC AAC AGG	24	3334	

2.4. Biochemical Tests

Biochemical tests, such as catalase, sugar fermentation (glucose, lactose, sucrose, and maltose), gelatin hydrolysis, mobility test, and indole analysis were performed for sample screening. Lecithinase activity of *C. novyi* isolates in the egg yolk agar plate was evaluated by anaerobically inoculating isolates by a sterile loop on the plate at 37˚C for 72 h (9).

3. Results

3.1. Isolation of the *Clostridium novyi* Isolate

After 72 h of anaerobic incubation, colonies showing irregular shapes with unclear borders appeared on agar media; moreover, Gram-positive, rod-shaped, and endospore-forming bacteria were identified in the colonies (Figure 1). Differential PCR was conducted on the DNA extracted from a single colony, which confirmed the isolate as *C. novyi*. No other bacterial and viral pathogens were detected.

3.2. Polymerase Chain Reaction

The positive control, *C. novyi* (CN804), strain was included in all PCR analyses, and it always proved positive as evaluated from electrophoresis in agarose gels. The PCR on the α-toxin produced a band in the range of 609 bp, indicating that the samples belonged to *C. novyi*. According to the PCR results obtained from α-toxin gene primers in type B *C. novyi*, 18 isolates of 75 suspected isolates contained the above gene from culture (Figure 2).

3.3. Biochemical Characterization of the *Clostridium novyi* Isolate

Molecular positive samples were confirmed by biochemical tests. Considering the morphology and biochemical characteristics, 18 isolates suspected of *C. novyi* were separated from 75 samples (Figure 3 and Table 2).

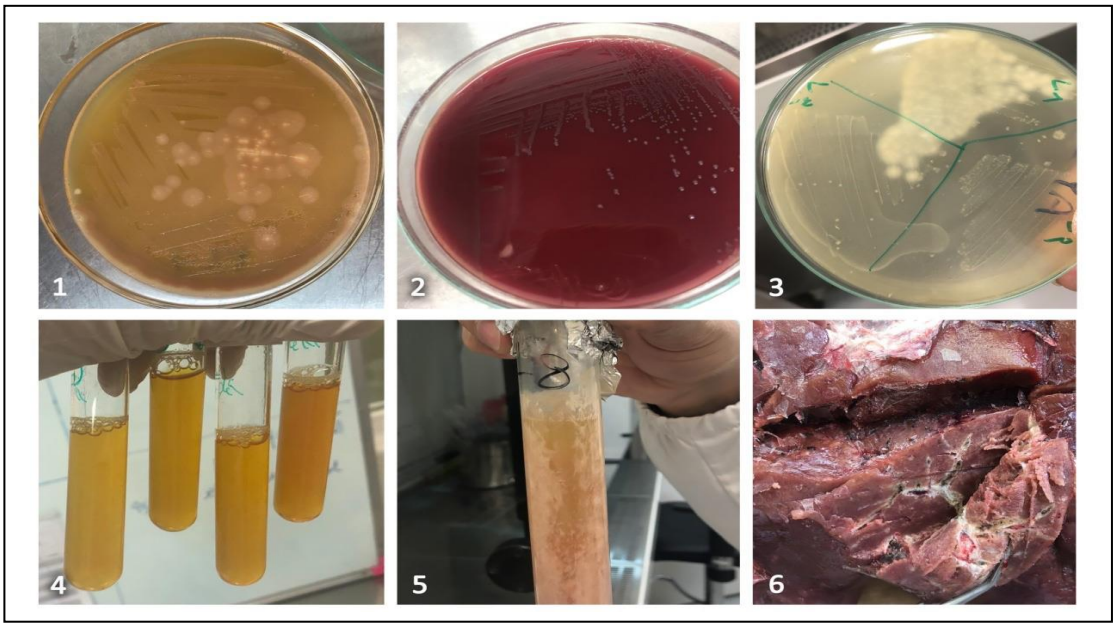

Figure 1. Clostridia medium under anaerobic conditions (Bacteria cultured in egg yolk agar medium [**1 and 3**], blood agar medium [**2**], *C. novyi* specific medium [**4**], chopped liver broth [**5**], liver parts were cut and crushed to obtain a bacterial sample [**6**])

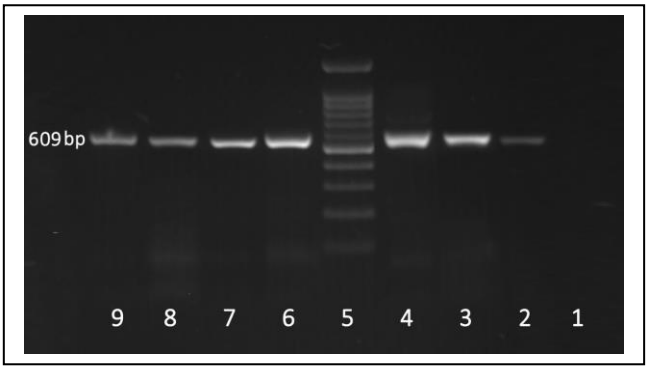

Figure 2. PCR of *Clostridium novyi* isolate (**1:** Negative control; **2:** Positive control; **3, 4, 6-9:** isolates; **5:** DNA size marker [100 bp, fermentas])

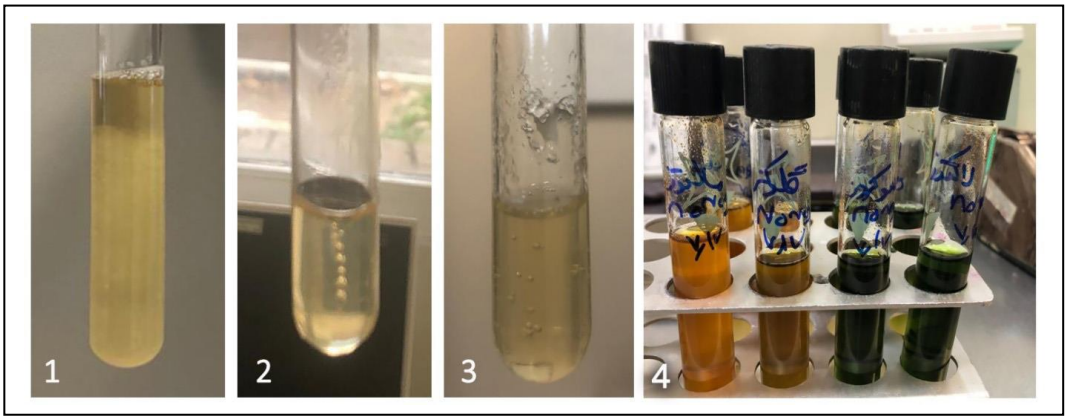

Figure 3. Biochemical characterization of the *Clostridium novyi* isolates (**1-** Growth in the anaerobic TSB thioglycolate; **2-** Motility test in ISM; **3-** Gelatin digestion, and 4- Sugar fermentation)

Table 2. Specifications of samples

Sample No.	Location	Sampling conditions	Liver condition	PCR results	Description
1	Mughan			Single band	Turbidity in culture
2					
3					
4			Healthy		No foam in culture
5	Shiraz	Clinical	Healthy	Non-specific bands	Low foam in culture
6					Turbidity in culture
7	Shahrekord	Clinical	Healthy		Low foam in culture
8	Ahvaz	Slaughterhouse	Healthy		Low foam in culture
9					
10					
11					
12					
13					Turbidity in culture
14					Low foam in culture
15					No foam in culture
16	Shahriar	Slaughterhouse	Healthy	Non-specific bands	Foam in anaerobic culture
17					No foam in culture
18					
19					
20				Main band and non-specific band	No foam in culture
21			Necrosis		
22					
23			Healthy	Main band and non-specific band	High foam in anaerobic culture
24			Low parasitic		
25			High parasitic infection of the larval migration route		
26			Parasitic		
27			High parasitic infection	Main band and non-specific band	No foam in culture
28			Healthy		
29	Marand	Slaughterhouse	Mild parasites		
30			Black parasite on the liver		Turbidity in culture
31			Healthy	Single band	
32			Healthy	Non-specific band slightly higher	Turbidity in culture, Slides and stained: Cocci and Clostridium
33			High parasitic infection	Single band	

Sample No.	Location	Sampling conditions	Liver condition	PCR results	Description
34				Single band	
35					
36					
37		Clinical	Suspicious		Turbidity in culture
38					
39					
40					
41			Healthy	Single band	
42					Turbidity in culture
43			Existence of calcification		
44				Non-specific band slightly higher	Turbidity in culture
45					
46					Turbidity in culture
47				Non-specific band	
48			Healthy	Main band and non-specific band	Foam in anaerobic culture
49				Main band and non-specific band	Turbidity in culture
50					
51				Non-specific band	Foam in anaerobic culture
52			Necrotic liver		
53	Ilam				Turbidity in culture
54					
55					
56		Slaughterhouse		Non-specific band	
57					
58					
59					
60				Single band	Turbidity in culture
61					
62					
63					
64					Turbidity in culture
65			Healthy		
66					No foam in culture
67					Turbidity in culture
68					
69					
70					
71					High foam in anaerobic culture
72					
73					
74					Foam in anaerobic culture
75					No foam in culture

4. Discussion

Clostridia can cause hepatic damage in domestic livestock, as well as wild and laboratory animals (10). *C. novyi* type B causes infectious necrotic hepatitis in sheep and less frequently in other species. These diseases cause heavy damages to sheep and goat herds (16). Vaccination is necessary to prevent clostridial diseases (17). To the best of our knowledge, there is no study on the isolation of *C. novyi* and molecular identification of α-toxin in Iran. These *C. novyi* isolates were first isolated from suspected sheep livers in Iran and confirmed by biochemical and molecular characterization.

Ardehali, Moosawi (18) isolated and identified *C. novyi* for the first time in 42 sheep liver samples suspected of Black disease collected from various regions in Iran. Out of collected samples, 27 positive samples were reported based on clinical findings after death and fluorescent antibody staining (immunofluorescence). Ardehali and Darakhshan (19) isolated *C. novyi* in 44 sheep liver samples suspected of Black disease based on fluorescent antibody staining. Isolate typing was performed based on lecithinase, hemolytic, necrotic, and lethal activity (20). Ardehali, Moosawi (21) studied the isolation, typing, and rapid identification of pathogenic clostridia isolated from infected animals. Smears were prepared from livers of sheep, goats, and cows suspected of Black disease, and after staining with a specific labeled antibody, 51 cases of *C. novyi* were identified by fluorescence microscopy. The typing of isolates was determined by the dermonecrotic test on guinea pigs and the lecithinase activity test (22).

According to the biochemical and molecular tests, such as PCR, 18 isolates were identified as *C. novyi*. Comparing the above results show that various techniques can be used to diagnose *C. novyi*; however, PCR seems more efficient and rapid than other techniques. Rapid testing seems important since *C. novyi* isolation and identification is rarely successful as samples should be quickly sent to the laboratory under anaerobic conditions.

Multiple isolations of sheep, cows, horses, and pigs have been performed by molecular and culture techniques worldwide. Sasaki, Takikawa (3) used the PCR technique based on 16S-23S rDNA regions for rapid differential identification of pathogenic clostridia involved in gas gangrene. Their results showed that the PCR patterns of 16S-23S rDNA regions could be used as a marker for the identification of pathogenic clostridia in gas gangrene (3). Jeong, Seo (9) identified a *C. novyi* case in sows (South Korea) by the simultaneous use of biochemical and molecular methods.

Due to the rapid post-mortem deterioration of tissues associated with many clostridial diseases, rapid preservation of tissue is required. Immunohistochemistry was invaluable in identifying the pathogen as no samples were taken for culture at post-mortem examination. Even the samples had been taken, the fastidious requirements of *C. novyi* often make successful culture difficult to achieve (23). Rapid post-mortem evaluation, histology, and immunohistochemistry were necessary to identify this pathogen and allow a presumptive diagnosis of Black disease. It is highly unlikely that an antemortem diagnosis would have altered the outcome of this case.

Of 75 isolates, 18 isolates were detected in the *C. novyi* PCR, indicating the excellent consistency of these two methods and the reliability of biochemical methods in determining the nature of this bacterium. Isolates were confirmed in this study by the biochemical method. There may be greater consistency between the mentioned techniques if more biochemical tests are used. Nonetheless, biochemical techniques for identifying *C. novyi* are time-consuming (24-72 h), and PCR is recommended in the cases where the detection speed is important due to the excellent consistency of these two methods.

Nyaoke, Navarro (16) investigated infectious necrotic hepatitis caused by *C. novyi* type B in a horse. In their study, liver samples tested by PCR were positive for *C. novyi* type B flagellin and α-toxin genes, but negative for *C. haemolyticum* and other clostridia.

In our study, additionally, a conventional PCR procedure was developed to amplify a segment of the *C. novyi* type B α-toxin gene, the main virulence factor of this microorganism.

After evaluating the genotype and phenotype characteristics of the isolates, the results of this study could reveal toxinogenesis and pathogenicity of isolates despite their genomic similarities, helping vaccine production from the vaccinal strain (from England) with at least 40-year history in Iran. The genetic and toxinogenesis comparison of isolated bacteria could be invaluable when the difference is reasonable.

There is no study on the *C. novyi* infection in the livestock in Iran by the culture and molecular confirmation techniques and vice versa. There is also no practical information for relevant organizations and vaccine manufacturers in Iran. In addition to evaluating and comparing these two identification techniques, the investigations of new isolates may help the vaccine industry in Iran.

Authors' Contribution

S. A. P. and M. N. designed and M. J. performed the experiment. A. E. and K. A. analyzed the data. S. A. P. and M. N. reviewed and edited the manuscript. A. E. wrote the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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