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NOTE

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Development of a novel *Trueperella pyogenes*-specific PCR assay

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ABSTRACT. *Trueperella pyogenes* is an opportunistic pathogen that causes a wide variety of purulent infections. We recently isolated a *T. pyogenes* strain unable to be identified by the previously reported *T. pyogenes* pyolysin gene (*plo*)-specific PCR from the lung of a sheep with astasia. Sequence comparison of *plo* among representative strains revealed several nucleotide substitutions in the primer-annealing regions. As such substitutions were considered to be a reason for the low PCR specificity, we designed novel primers in conserved regions of *plo*. Under optimized conditions, the novel primers precisely identified all *T. pyogenes* strains tested, and no products were generated from any other bacterial strains, suggesting the usefulness of the novel PCR assay for the diagnosis of *T. pyogenes* infections.

KEY WORDS: pyolysin gene, specific PCR, *Trueperella pyogenes*

Trueperella pyogenes is a gram-positive, nonmotile, nonsporulating and short rod-shaped facultative anaerobic organism that exhibits hemolytic activity on blood agar [5]. It belongs to the family *Actinomycetaceae* and was previously classified as *Corynebacterium pyogenes*, *Actinomyces pyogenes* and *Arcanobacterium pyogenes* [5]. *T. pyogenes* is known to constitute a part of the microbiota, and can be found on the skin, mucous membranes of the upper respiratory and urogenital tracts, the walls of the rumen and stomachs, and the udders of healthy animals. This bacterium is also known as an important opportunistic pathogen that causes a wide variety of purulent infections in livestock, including pneumonia, liver abscessation, metritis, mastitis, endocarditis, pleuritis, osteoarthritis, polyarthritis and septicemia [5]. *T. pyogenes* infections often cause significant economic losses in livestock industries [5], and accurate diagnosis including precise identification of the causative agent is a prerequisite for reducing such losses.

T. pyogenes is usually identified based on colony and cell morphology, and biochemical characteristics. However, the identification of *T. pyogenes* by these characteristics takes several days. In addition, for the differentiation and precise identification of isolates, molecular techniques may be needed [5]. Although recent techniques, such as loop-mediated isothermal amplification (LAMP) assay, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, Fourier transform infrared (FT-IR) spectroscopy and 16S rRNA gene sequencing, may be available for this purpose [5], PCR, which is widely employed and can produce results in a relatively short time, is also useful.

A PCR assay targeting the *T. pyogenes*-specific hemolysin (pyolysin) gene (*plo*) was reported and has been utilized to identify *T. pyogenes* strains [2, 3]. However, we recently found a *T. pyogenes* strain that cannot be identified precisely by this previously reported *T. pyogenes*-specific PCR (original *plo*-PCR). In this study, we analyzed the strain in detail, including its *plo* gene, and developed a novel PCR assay that can identify the strain as *T. pyogenes* unambiguously.

The strain (designated as DTK435) was isolated from the lung of a sheep in 2018. The sheep developed pneumonia in May and was suspected of having mannheimiosis. Although the sheep recovered, it exhibited convulsions and astasia in early June, and was considered to have a poor prognosis. DTK435 was a catalase-negative and oxidase-negative Gram-positive rod, and exhibited complete hemolysis on blood agar media. The strain was identified as *T. pyogenes* when tested by API Coryne (bioMérieux, Marcy-l'Etoile, France), a biochemical test kit to identify *Corynebacterium* and coryne-like organisms (Supplementary Table 1). However, when the original *plo*-PCR [3] was performed according to the conditions described in the report in a 20-*μl* reaction volume using 100 *n*g of the genomic DNA extracted by InstaGene Matrix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and

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(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

Table 1. *Trueperella pyogenes* pyolysin gene (*plo*)-specific PCR primers designed in this and previous studies

Primer	Sequence (5′-3′)	PCR product size	Source or reference	Polymerase used for PCR	$MgCl_2$ concentration	Primer concentration	PCR program
ploF[a)]	GGCCCGAATGTCACCGC	270 bp	3	Ex Taq (Takara Bio Inc., Kusatsu, Japan)	1.5 mM	0.5 μM	94°C 2 min 94°C 1 min, 55°C 1 min, 72°C 1 min (35 cycles) 72°C 5 min
ploR[a)]	AACTCCGCCTCTAGCGC			KOD FX (TOYOBO, Osaka, Japan)	2 mM	0.3 μM	94°C 2 min 98°C 10 sec, 55°C 1 min, 68°C 1 min (35 cycles) 68°C 5 min
ploNF	AACGGCCTTCTCGACGGTTG	493 bp	This study	KOD FX (TOYOBO)	2 mM	0.3 μM	94°C 2 min 98°C 10 sec, 68°C 30 sec (30 cycles) 68°C 2 min
ploNR	TAGCTCGGGTCTTGTTCAGG			KOD One (TOYOBO)	2 mM	0.3 μM	98°C 10 sec, 68°C 5 sec (30 cycles)

a) Primer names were designated in this study.

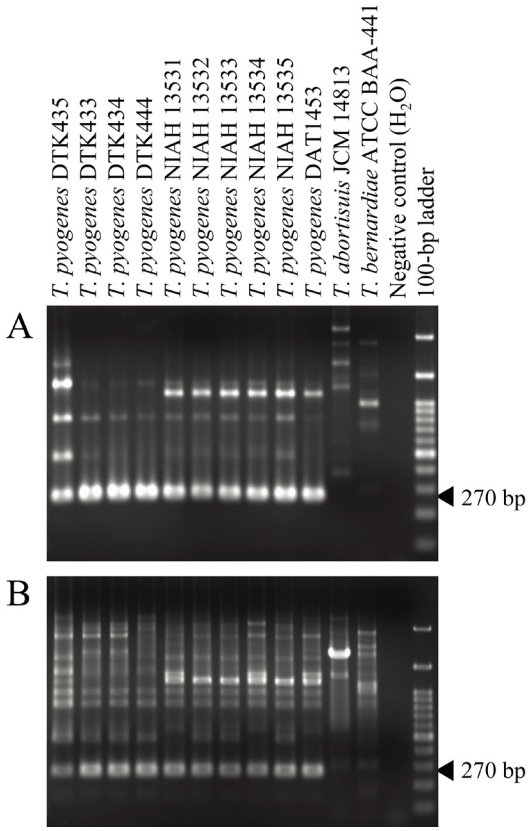

Fig. 1. Results of the *Trueperella pyogenes* pyolysin gene (*plo*)-specific PCR assay (original *plo*-PCR) reported previously. DNA samples were extracted from each bacterial strain grown on appropriate agar media by InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA), and 100 *n*g was used as the template for each reaction. *TaKaRa Ex Taq* (Takara Bio Inc., Kusatsu, Japan) (A) and KOD FX (TOYOBO, Osaka, Japan) (B) were used for the reactions, and the PCR conditions are shown in Table 1. Five microliters of PCR product was run on a 1.5% agarose gel and stained with ethidium bromide.

DNA polymerases listed in Table 1, many nonspecific products were amplified from DTK435 (Fig. 1). Thus, we were unable to conclude whether DTK435 was *T. pyogenes* by these tests. For precise identification, we then determined the 16S rRNA gene sequence of DTK435 according to the methods described previously [1], and pairwise similarities between the 16S rRNA gene sequences of DTK435 and the type strain of each species were calculated using the EzBioCloud server (https://www.ezbiocloud.net) [6]. The 16S rRNA gene sequence of DTK435 shared 100% identity with that of the type strain of *T. pyogenes*, but 98.59% or less identity with those of the other species. This strongly suggested DTK435 to be *T. pyogenes*.

We performed the original *plo*-PCR under other conditions (different annealing temperatures [57.3°C, 58.7°C and 61°C], extension times [20 sec] and amount of template DNA [50 *n*g in a final reaction volume of 20 μl]); however, PCR results for DTK435 were not improved. Even under high annealing temperature (61°C) and 50 *n*g template DNA conditions, non-specific products were still

Table 2. Bacterial strains used in this study

Bacterial species/strain	Origin/reference	Accession no. of 16S rRNA gene sequences determined in this study	Accession no. of *plo* gene sequences determined in this study
Trueperella pyogenes DTK435	Lung of diseased sheep	LC500004	LC500001
Trueperella pyogenes DTK433	Pons of goat	LC500005	
Trueperella pyogenes DTK434	Abscess in the goat brain	LC500006	LC500002
Trueperella pyogenes DTK444	Lung of diseased cattle	LC500007	
Trueperella pyogenes NIAH 13531	Abscess in the cerebellum of swine	LC500008	
Trueperella pyogenes NIAH 13532	Lung of diseased swine	LC500009	
Trueperella pyogenes NIAH 13533	Brain of diseased swine	LC500010	
Trueperella pyogenes NIAH 13534	Lung of diseased swine	LC500011	LC500003
Trueperella pyogenes NIAH 13535	Abscess in a hind leg of swine	LC500012	
Trueperella pyogenes DAT1453	Ileum of desease swine	LC500013	
Trueperella abortisuis JCM 14813	Sow placenta after abortion, Japan Collection of Microorganisms (JCM)	LC500014	
Trueperella bernardiae ATCC BAA-441	Fluid from knee culture, surgical site, American Type Culture Collection (ATCC)	LC500015	
Actinobacillus pleuropneumoniae 4074 (type strain)	Lung of swine		
Actinobacillus pleuropneumoniae ATCC 27090	Periarticular abscess of swine, ATCC		
Escherichia coli DAT1476	Diarrhea of swine		
Haemophilus parasuis Nagasaki	Swine with Glasser's disease		
Haemophilus parasuis SW140	Nasal cavity of swine		
Histophilus somni 8025 (type strain)	Brain of cattle with thromboembolic meningoencephalomyelitis		
Mannheimia haemolytica ATCC 33396 (type strain)	Sheep, ATCC		
Mannheimia haemolytica ATCC 43270	Bovine pneumonia, ATCC		
Pasteurella multocida ATCC 43137 (type strain)	Swine, ATCC		
Pasteurella multocida ATCC 43019	Bovine, ATCC		
Pasteurella multocida BP174	Bovine pneumonia		
Pasteurella multocida SP-72	Swine		
Salmonella Newport DTK223	Loose stool of bovine		
Actinomyces hyovaginalis DTK445	Diseased cattle, lung, co-isolated with *T. pyogenes* DTK444		
Arcanobacterium pluranimalium DTK431	Bovine abortion		
Clostridium chauvoei Okinawa	Vaccine strain		
Clostridium perfringens CP-23	Bovine		
Clostridium perfringens NCTC 3227	The National Collection of Type Cultures (NCTC)		
Clostridium septicum ATCC 12464 (type strain)	ATCC		
Corynebacterium bovis DTK382	Bovine mastitis		
Erysipelothrix rhusiopathiae ATCC 19414 (type strain)	Spleen of pig with endocarditis, ATCC		
Erysipelothrix rhusiopathiae Fujisawa	Swine with acute septicemia		
Facklamia sourekii DAT97	Bovine hematuria		
Helcococcus kunzii DTK446	Diseased cattle, lung, co-isolated with *T. pyogenes* DTK444		
Staphylococcus aureus DAT897	Bovine mastitis		
Streptococcus pluranimalium DAT1470	Nasal cavity of healthy cattle		
Streptococcus ruminantium DTK394	Bovine pneumonia		
Streptococcus suis P1/7	Swine with meningitis		

well amplified, whereas signal intensity of the specific 270-bp product on agarose gel became weak (data not shown). Therefore, we evaluated the specificity of the PCR using nine *T. pyogenes* strains in our laboratory collection and strains of two other *Trueperella* species (*Trueperella abortisuis* JCM 14813 and *Trueperella bernardiae* ATCC BAA-441) (Table 2). The 16S rRNA gene sequences of the nine *T. pyogenes* strains shared 99.62–100% identity with that of the *T. pyogenes* type strain. These nine strains were also identified as *T. pyogenes* by API Coryne (bioMérieux) (Supplementary Table 1). On the other hand, 16S rRNA gene sequences of *T. abortisuis* JCM 14813 and *T. bernardiae* ATCC BAA-441 shared only 98.63% and 97.79% identity, respectively, with that of the *T. pyogenes* type strain; therefore, these two species can be distinguished from *T. pyogenes* using 16S rRNA gene sequences. However, when tested by API Coryne (bioMérieux), *T. abortisuis* was misidentified as *T. pyogenes* (Supplementary Table 1), suggesting that appropriate molecular diagnostic tools are needed for reliable identification of *T. pyogenes*. Furthermore, the original *plo*-PCR primers

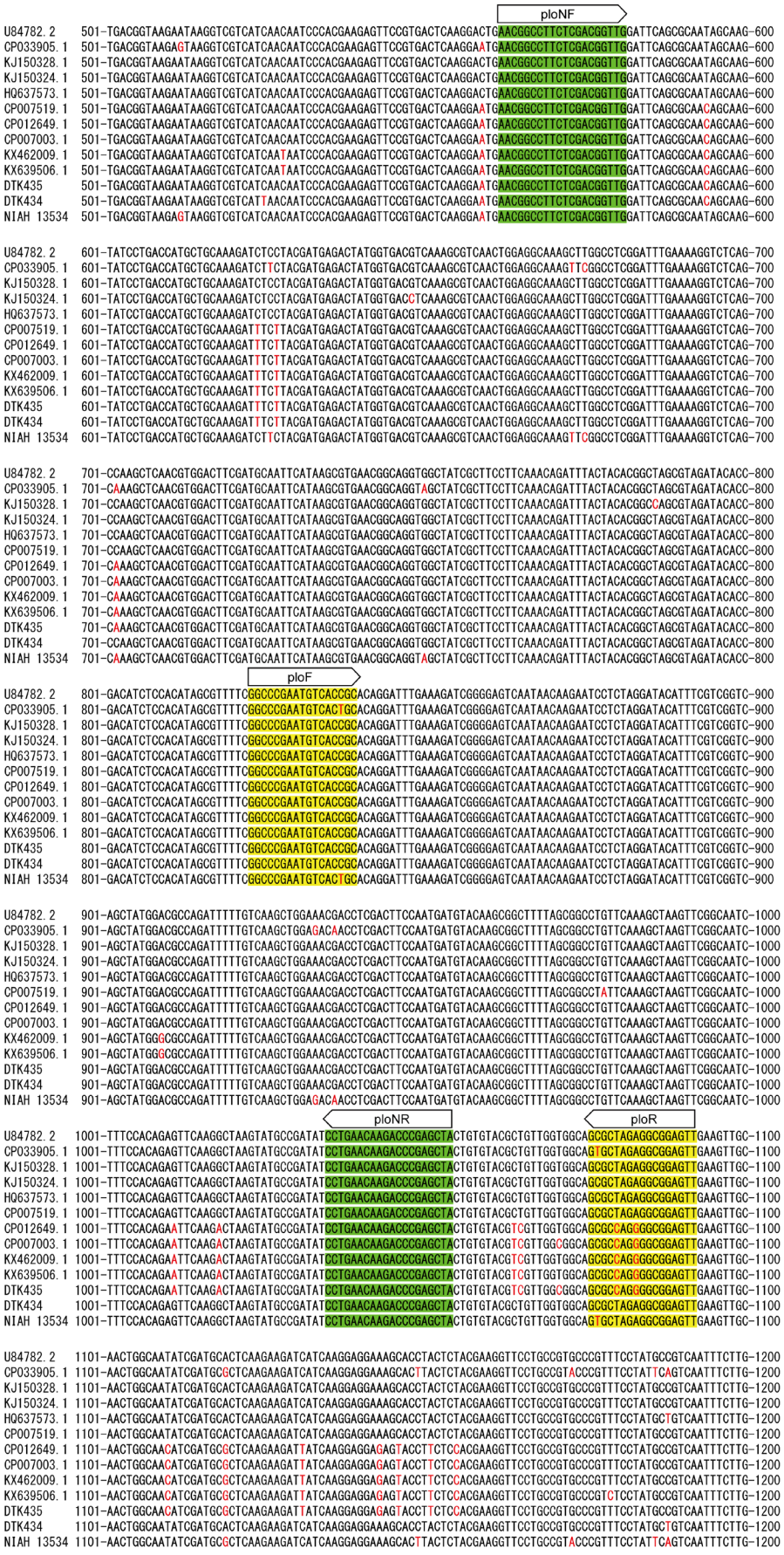

Fig. 2. Multiple nucleotide sequence alignment of *Trueperella pyogenes* pyolysin genes (*plo*). The *plo* gene sequences retrieved from the GenBank database were compared with those of *T. pyogenes* DTK435, DTK434 and NIAH 13534. The multiple alignment was computed using ClustalW (https://clustalw.ddbj.nig.ac.jp/). In the case that two or more identical sequences were found in the database, a representative sequence was selected and used for this figure. The numbers indicate nucleotide positions in the *plo* genes. Accession nos. of the retrieved sequences are shown in this figure, and nucleotides different from the top sequence (accession no. U84782.2) are shown in red letters. The positions of the *plo* gene-specific primers designed in this (ploNF and ploNR) and the previous (ploF and ploR) [3] studies are indicated above the sequences.

and conditions yielded many nonspecific PCR products from all the *Trueperella* strains used in this study (Fig. 1).

In order to investigate the cause of the low specificity of the *T. pyogenes*-specific primers (ploF and ploR), we selected DTK434, DAT435 and NIAH 13534 as representative strains, and amplified their partial *plo* gene regions by PCR using the primers and conditions listed in Supplementary Table 2 in the online Supplementary Material. The amplified products were purified by the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and their sequences were determined using the BigDye Terminator v3.1 cycle sequencing kit and 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Primers used for the sequencing are listed in Supplementary Table 2. Comparison of the determined *plo* sequences and those retrieved from the GenBank database revealed the presence of many nucleotide substitutions (Fig. 2). Such substitutions were also present in the *T. pyogenes*-specific primer binding sites (Fig. 2), and may have been one of the causes of the low specificity of the primers. In addition to the specific primer binding sites, other sequences homologous to the primer binding sites may exist in the genomes of *T. pyogenes* strains, and non-specific binding of the *plo* primers to such sequences may cause non-specific amplification. Some of the faint non-specific products observed in this study may decrease under high annealing temperature conditions. However, as described above, PCR results for DTK435 were not improved even under high annealing temperature conditions. Therefore, to develop a novel *plo*-PCR that can identify *T. pyogenes* unambiguously, we designed primers ploNF and ploNR in the regions conserved among all *plo* genes analyzed in this study (Fig. 2).

Under optimized conditions using KOD FX (TOYOBO Co., Ltd., Osaka, Japan) (Table 1), the new primers yielded only a single specific PCR product of expected size from all *T. pyogenes* strains used in this study in approximately 65 min, and no products were generated from any other bacterial strains tested, including other *Trueperella* species and those isolated from diseased animals (Table 2 and Fig. 3). When the PCR was performed using KOD One (TOYOBO) under the conditions listed in Table 1, the specific products were amplified sufficiently in approximately 30 min (Supplementary Fig. 1 in the online Supplementary Material). Furthermore, the novel *plo*-PCR detected *T. pyogenes* DTK433 from 10 *p*g of genomic DNA (Fig. 4).

For reliable identification of *T. pyogenes*, molecular diagnostic tools in addition to biochemical tests are necessary. In this study, through the discovery of a *T. pyogenes* strain that was unable to be identified by the original *plo*-PCR, we developed a novel *plo*-PCR with high specificity. In the genus *Trueperella*, in addition to the three *Trueperella* species used in this study, there are two more species (*Trueperella bialowiezensis* and *Trueperella bonasi*). Although we have not evaluated specificity of the novel *plo*-PCR using these two species, they have so far been isolated only from preputial swabs of European bison bulls [4], and no isolation from domestic animals was reported. Therefore, our novel PCR is considered to have enough specificity for practical use in the field of livestock hygiene. High sensitivity is not necessarily needed because it is assumed to be used using isolated strains; however, our PCR also demonstrated relatively high sensitivity. As many nucleotide substitutions were observed in the *plo* genes analyzed in the present study, we cannot rule out the possibility that the specificity of our PCR will decrease in the future due to additional nucleotide substitutions in the target gene. However, our newly developed *T. pyogenes*-specific PCR will be a useful tool for the rapid and reliable identification of this important opportunistic pathogen.

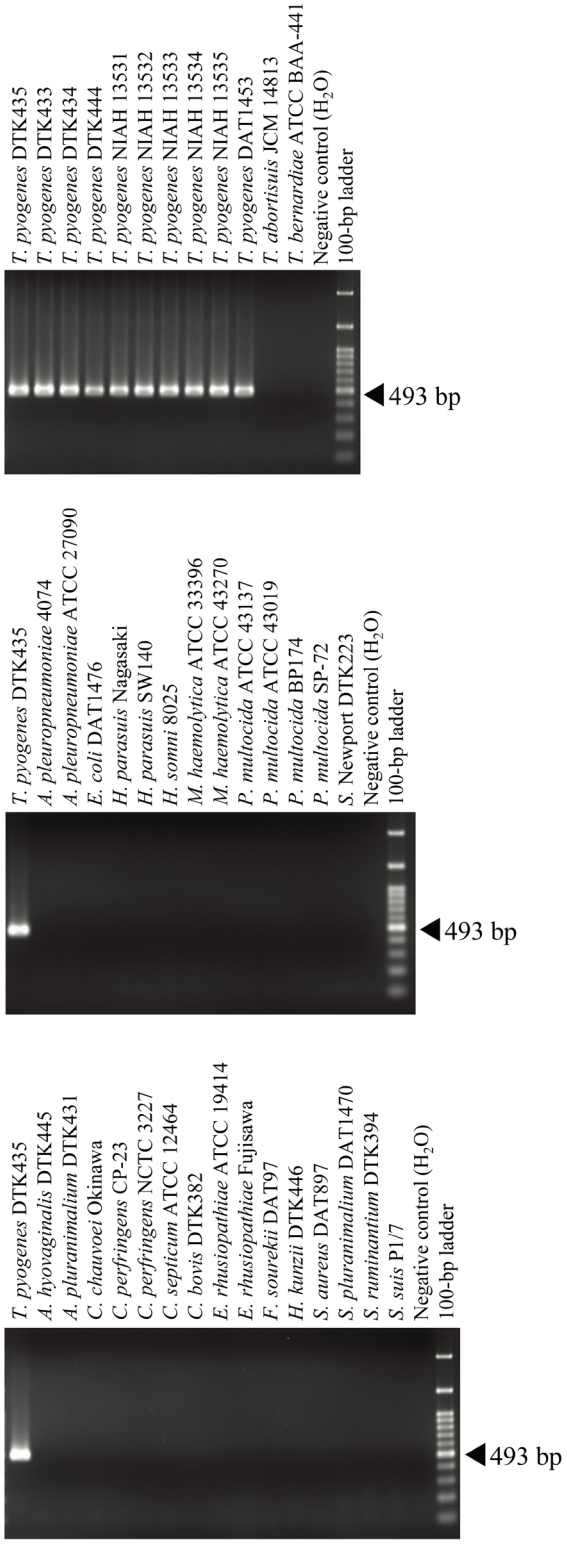

Fig. 3. Specificity of the novel *Trueperella pyogenes* pyolysin gene (*plo*)-specific PCR assay (novel *plo*-PCR) developed in this study. DNA samples were extracted from each bacterial strain grown on appropriate agar media by InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA), and 100 *n*g was used as the template for each reaction. KOD FX (TOYOBO, Osaka, Japan) was used for the reactions, and the PCR conditions are shown in Table 1. Five microliters of PCR product was run on a 1.5% agarose gel and stained with ethidium bromide.

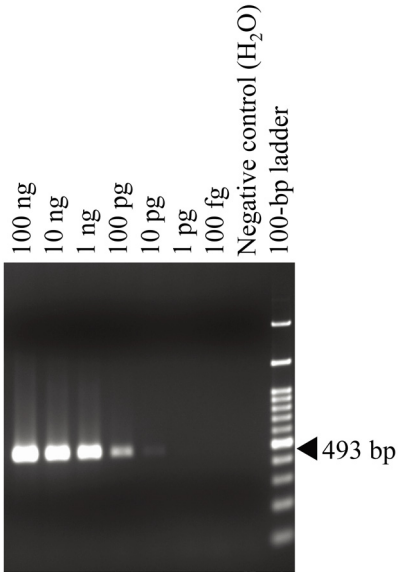

Fig. 4. Sensitivity of the novel *Trueperella pyogenes* pyolysin gene (*plo*)-specific PCR (novel *plo*-PCR). Serial dilutions of DNA extracted from *T. pyogenes* DTK433 by InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) were used to investigate the sensitivity of the PCR. The amount of genomic DNA used as the template for each reaction (final reaction volume, 20 *µl*) is indicated above each lane. KOD FX (TOYOBO, Osaka, Japan) was used for the reactions, and the PCR conditions are shown in Table 1. Five microliters of PCR product was run on a 1.5% agarose gel and stained with ethidium bromide.

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