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New methods for isolation of keratolytic bacteria inducing intractable hoof wall cavity (*Gidoh*) in a horse; double screening procedures of the horn powder agar-translucency test and horn zymography

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To establish a new system to isolate keratolytic bacteria from the hoof wall cavity (Gidoh) of a racehorse, we invented the horn powder agar-translucency (HoPAT) test and horn zymography (HZ). Using routine bacteriological techniques and these methods, we isolated five strains of keratolytic soil bacteria, which were then identified by means of 16S ribosomal RNA (rRNA) gene sequencing analysis. The findings from the study on the horse suggested that Brevibacterium luteolum played the main role in the local fragility of the hoof, eventually forming a Gidoh in coordination with four other strains of keratolytic bacteria. The double screening procedures of the HoPAT test and HZ were useful and easy techniques for isolating the keratolytic bacteria from the horn lesions.

Key words: *Gidoh, hoof, horse, keratolytic bacteria*

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A hoof wall cavity (*Gidoh*) is frequently formed between the *stratum medium* and the *stratum internum* in the forefeet of racehorses [16, 20]. Because a *Gidoh* is usually opened to the bearing surface and comes into direct contact with the ground, it can easily be filled with soil, which includes a wide variety of soil microorganisms. Thus, soil fungi and/or bacteria have routinely been isolated from lesions of the *Gidoh*. Many direct or supporting lines of evidence have indicated that possible contributors to the formation of the *Gidoh* include decreasing toughness of the wall based on abnormal anatomical conformation of the foot [2, 18], swelling and softening of the horny structure by water and/or urine [6–9, 19], neglect of incipient lesions relating to irregular farriery [2], and infection of the wall by microorganisms [1, 2, 4, 5, 12, 13, 15–17, 19]. Scientific research data have shown that keratinopathogenic fungi can invade into, and proliferate in, horny structures of the foot [1, 15, 17]. This can result in the collapse of the hoof capsule due to the production of keratolytic enzymes [1, 12]. Regardless of many reports of bacteria within wall lesions, no report has as yet scientifically proven that the bacteria isolated from a hoof wall cavity can produce keratolytic enzymes that invade the horn and eventually disrupt the hoof wall. Therefore, the development of a new method is required to discriminate bacterial infestation from simple contamination with soil in the *Gidoh*. To discriminate between these conditions, it is logical to assess whether the isolated bacteria show keratolytic activity.

We encountered a racehorse suffering from recurrent *Gidoh* without a history of laminitis. By means of a combination of two newly developed procedures, 1) a horn powder agar-translucency (HoPAT) test and 2) horn zymography (HZ), we attempted to prove that keratolytic bacteria were associated with the *Gidoh*. Furthermore, we attempted to identify the isolated keratolytic bacteria using 16S ribosomal RNA (rRNA) gene sequencing analysis. The present report is the first study to provide direct scientific evidence of the existence and involvement of keratolytic bacteria in the *Gidoh*.

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Materials and Methods

Horse history

The studied horse (thoroughbred, male, six years old) was retired from racing due to the recurrent white line disease-type *Gidoh* [16, 20] manifesting on the forefeet with no response to regular shoeing, corrective farriery, and local debridement. At the first examination after retirement, we identified an obsolete dry cavity in the wall of the right forefoot and a fresh moist cavity in the wall of the left forefoot. The latter cavity showed fine branching lines that ran between the *stratum medium* and the *stratum internum* of the wall from the bearing surface of the white line to 5 cm above the bearing border (Fig. 1).

Fig. 1. A fragile area of the horn was observed at the toe of the left foot. A progressive fine linear cavity ran through the middle of the fragile lesion. In this picture, a cross-sectional surface of the cavity shows a punctate hole between the *stratum medium* and the *stratum internum*.

Sampling

We investigated the fresh progressive wall lesion of the left foot in the present study. Horn pieces of 1 mm in width and 1–3 mm in length were resected with a loop hoof knife (Imai Inc., Nagoya, Japan) from the highest lesion along the side of the cavity. After washing three times with sterile phosphate-buffered saline (PBS; pH 7.6), each horn piece was divided into two pieces for pathological diagnosis and bacteriological examination.

Pathological diagnosis

Immediately after washing, pathological samples were sectioned under cooling conditions (−20°C) with a cryosection machine (Cryostat, CM3050, Leica Microsystems K.K., Tokyo, Japan) without any chemical fixation or liquid nitrogen freezing. Each sample was sliced into 10-μm-thick sections by a sledge-type cryotome. Three equivalents of each section were stained with hematoxylin and eosin (HE), Gram, and Periodic acid-Schiff (PAS) stains with floating staining procedures using 10-mm diameter disk-type of stainless steel mesh baskets (Sanshin Industrial Co., Ltd., Yokohama, Japan). Consequent to dehydration with a dilution series of alcohol and xylene, each stained section was mounted with Eukitt mounting medium (ORSAtec GmbH, Bobingen, Germany).

Bacterial isolation

A volume of 0.01 g/m*l* of each sample for bacteriological examination was homogenized in PBS using a disposable tissue grinder (Fisherbrand, Fisher Scientific, TX, U.S.A.). Hundred-fold serial dilutions (×100 to 10,000) of the homogenate suspension were cultured (24 hr/37°C) on 5% horse blood-supplemented Colombia agar plates with addition of amphotericin B (Fungizone, Life Technologies, Tokyo, Japan) under aerobic and anaerobic conditions to isolate indwelling bacteria. After subculture of each isolated dominant bacterium, each bacterium was re-cultured with Heart Infusion Broth (Nissui, Pharmaceutical Co., Ltd., Tokyo, Japan). The medium was then frozen at −80°C after supplementation with 10% dimethyl sulfoxide until usage.

Preparation of horn powder-mixed agar plates

For biochemical analysis of the keratolytic activity of the bacteria, we prepared horn powder using an adequate amount of hoof wall collected from five horses (males, 3–6 years old) euthanized because of catastrophic leg injuries. Each hoof wall was cut into 5-mm cuboidal pecies and frozen in a deep freezer (−30°C), and they were then ground with a stainless steel mill-type cryogenic grinding machine (TI-500DX/ET, Cosmic Mechanical Technology Co., Ltd., Fukushima, Japan). Subsequently, most blood components and water-soluble non-keratinous proteins in these ground horny tissues were removed by dissolution using 6 M guanidine hydrochloride with 0.1% 2-mercaptoethanol and addition of proteinase inhibitor cocktail (Complete Ultra Tablets, Roche Diagnostics GmbH, Mannheim, Germany). The ground horny tissues were then dialyzed using a cellulose membrane (Cellulose tube TM, Sanko-Seiyaku, Ltd., Tokyo, Japan) with a 50:1 MilliQ water:dissolved horn material volume ratio. After centrifugation (4°C, 3,000 rpm, 5 min), the precipitate was lyophilized by a freeze-drying device (Freezemobile FM8SL, Virtis, U.K.) and stored under freezing conditions (−30°C) until use. When needed, the lyophilized horn powder was reground with a pestle and mortar to achieve a consistent quality, and subsequently sterilized with ethanol (30 min). After removing ethanol

by centrifugation and suction, we alternatively mixed the MilliQ water into the lyophilized horn powder with agitation using an electric stirrer. The suspension of the horn powder with the MilliQ water was mixed with autoclaved nutrient agar solution (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) containing 0.25% amphotericin B (Fungizone, Life Technologies, Tokyo, Japan) to obtain a final concentration of horn powder of 0.5% in terms of volume rate. All suspensions were mixed by stirring in a hot water bath at 50°C. Thus, we prepared plastic plates (φ 18 cm) filled with the agar containing the equine hoof-horn powder. We selected only plates that showed a uniformly clouded white color, positively indicating the presence of insoluble horn powder.

Preparation of polyacrylamide gel to blend crystallized water-soluble horn for electrophoresis

The lyophilized horn powder prepared in the preceding protocol was suspended in a 100-fold volume of 0.01 M sodium hydroxide and then hydrolyzed using an autoclaving device (121°C, 5 min). After cooling and centrifugation (4°C, 30,000 rpm, 10 min), the supernatant was dialyzed following lyophilization to prepare the crystallized water-soluble equine horn. We assessed whether the antigenicity and molecular weights of the crystallized water-soluble horn corresponded to those of reported equine hoof wall cytokeratins (47–71 kD) [25] using routine procedures of western blotting with anti- pan-cytokeratin antibody (AE1/AE3, DAKO Japan Inc., Tokyo, Japan) and 3,3′-diaminobenzidine. Following confirmation of preservation of cytokeratin skeletons within the crystallized water-soluble horn, we prepared separating gels of 40% acrylamide, into which 10 mg of the water-soluble equine horn was impregnated (final concentration, 0.16%) for conduct of horn zymography by electrophoresis. In addition, stacking gels of 5% acrylamide were prepared according to general procedures.

Primary screening examination by horn powder agar-translucency (HoPAT) test

The isolated dominant bacteria were subcultured on the aforementioned equine horn powder agar plates at 37°C overnight. Only bacteria showing translucency around colonies on the agar were recognized as having passed through the first filter of this keratolytic activity test.

Secondary screening examination by horn zymography (HZ) electrophoresis

For the purpose of excluding false positive keratolytic bacteria from the HoPAT test, we conducted HZ as a secondarily screening examination. The bacteria found to be positive in the HoPAT test were re-cultured at room temperature overnight in Heart Infusion Broth (Nissui Pharmacertical Co., Ltd., Tokyo, Japan). After centrifugation (1,000 rpm, 10 min), sodium dodecyl sulfate (SDS) sample buffer was added to each supernatant (New England BioLabs Japan, Tokyo, Japan), after which two-dimensional gel electrophoresis was conducted using the previously prepared acrylamide gel combined with the crystallized water-soluble equine horn. Thus, electrophoresis to separate keratolytic enzymes was implemented with a size marker of the protein ladder (10–250 kD; P7703S, New England Biolabs Inc., Hitchin, U.K.). This electrophoresis procedure was conducted with Tris-glycine buffer (Tris 30 g, Glycine 144 g, and SDS 5 g in 5 *l* of MilliQ water) at room temperature without boiling the samples to avoid deactivation of the keratolytic enzymes. After electrophoresis, each gel was washed in 2.5% Triton X-100 and then soaked in a prepared buffered solution (5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 200 mM NaCl in 50 mM Tris-HCl; pH 7.6) to activate the enzymatic reaction at room temperature overnight. Subsequently, all activated gels were stained with 0.1% Coomassie Brilliant Blue (CBB) R250/25% isopropyl alcohol to assess keratolytic activity. If a transparent or translucent band appeared in the background stained with CBB, we determined those bacteria to show a positive reaction to HZ, thereby positively showing keratolytic activity.

Bacterial identification based on 16S rRNA gene analysis

Bacterial identification was conducted according to previously published methods of 16S rRNA gene sequencing analysis [10]. Total DNA of the bacteria that showed positive reactions to both of the HoPAT test and HZ were extracted using InstaGene^{TM} Matrix (Bio-Rad Japan, Tokyo, Japan) according to the manufacturer's instructions. Full-length16S rRNA gene fragments from the extracted DNAs were amplified by polymerase chain reaction (PCR) with universal primers (forward 27f: 5′-AGA GTT TGA TCC TGG CTC AG-3′, reverse 1492r: 5′-GGT TAC CTT GTT ACG ACT T-3′) using commercial master mix (SapphireAmp Fast PCR Master Mix, Takara BIO Inc., Shiga, Japan). These amplified PCR products were purified with a QIAquick PCR Purification Kit (Qiagen Co., Ltd., Tokyo, Japan). After confirming molecular sizes of the obtained amplicons by means of routine procedures using agarose gel electrophoresis, sequencing analysis using synthesized universal primer sets, the forward and reverse primers of which were f1L, f2L, 926f, and f3L and r1L, r2L, r3L, and r4L, respectively [11, 24], was performed by a commercial service (FASMAC Co., Ltd., Atsugi, Japan). Homology analysis was performed using a Basic Local Alignment Search Tool (BLASTN) search (http://blast.ncbi.nlm.nih.gov/) of the published 16S rRNA gene sequences in GenBank. The species or genus of the isolates was defined according to the criteria of the Clinical and Laboratory

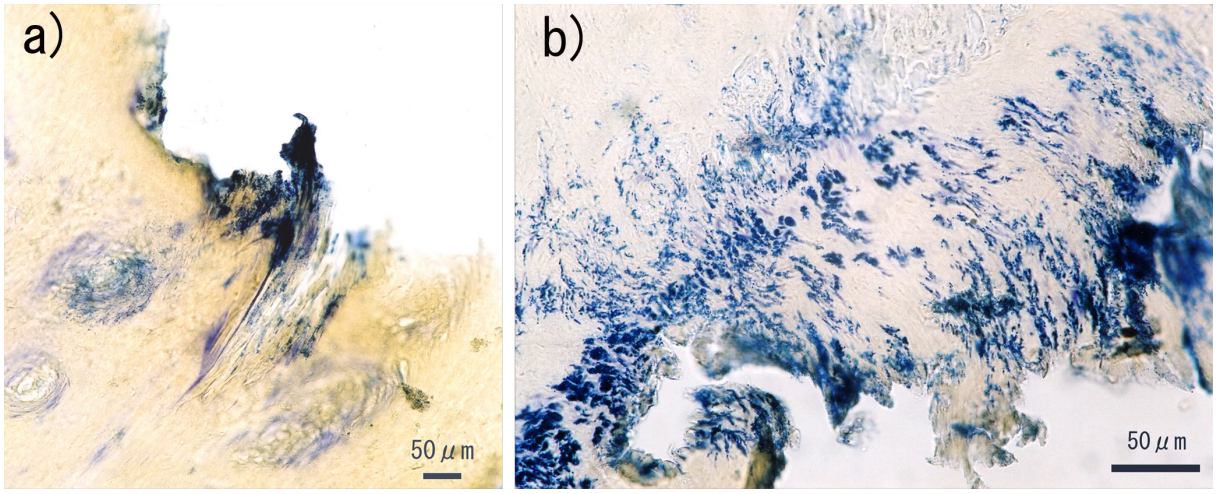

Fig. 2. A large number of Gram-positive bacteria were attached to the border of the hoof wall cavity (a) and invaded and proliferated inside the horn tissues near the cavity (b). All bacteria were observed within 300 μm from the edge of the lesion in the depth direction.

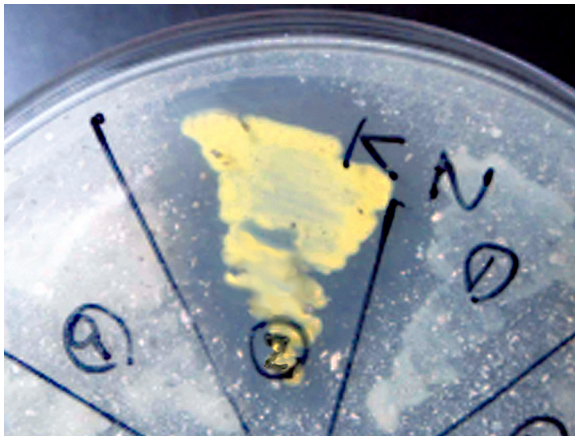

Fig. 3. One of the culture results from the horn powder agar-translucency test: The strain in ②(corresponding to No. 2) appeared as a yellowish colony and presented a stronger and wider translucency characteristic around the colony on the horn powder agar than other strains. The colony in ①on the right side of No. 2 showed weak translucency, whereas ⑨had no translucency characteristic.

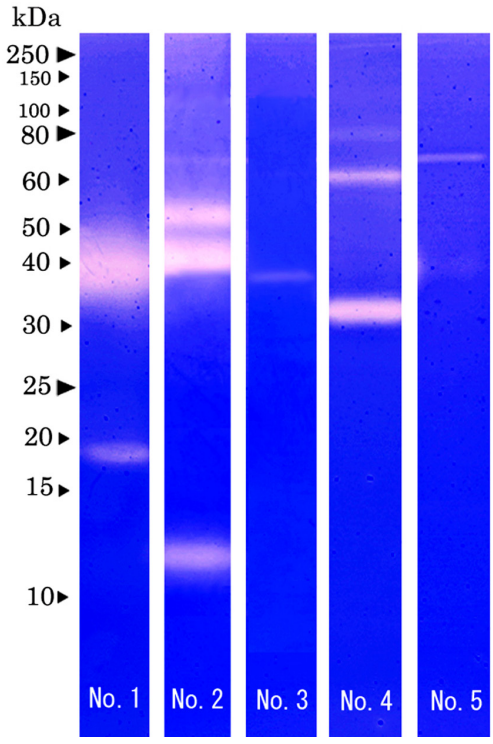

Fig. 4. In the horn zymography, the keratolytic activity of each isolated bacterium (from No. 1 to No. 5) was visible as one to four white bands in the water-soluble horn-combined acrylamide gel stained with Coomassie Brilliant Blue (CBB) in the secondary screening examination.

Standards Institute (CLSI). In other words, we identified a bacterium with ≥99.0% identity to the type strain as one corresponding to the species level, one with ≥97.0% to <99.0% identity to the type strain as one corresponding to the genus level, and one with <97.0% identity to the type strain as one corresponding to a closely related genus or possibly to a novel bacterium.

Table 1. Characteristics of keratolytic bacteria isolated from the "*Gidoh"*: the hoof wall cavity of a racehorse

Sample number	Morphology of microbes	Gram stain	HoPAT test	Number of keratinolytic enzymes	Molecular sizes of keratinolytic enzymes	Most homologus type strain	Accession number of type strain	Identity (homologus rate) to type strain (%)
1	Cocci or small bacilli	Positive	Weak	2	39, 18	*Dermabacter hominis*	X91034	96
2	Bacilli	Positive	Strong	4	68, 56, 40, 13	*Brevibacterium luteolum*	AJ488509	100
3	Small bacill	Positive	Weak	1	36	*Corynebacterium freneyi*	AJ292762	99
4	Small bacill	Positive	Weak	3	80, 70, 35	*Agrococcus casei*	DQ168427	98
5	Bacilli	Positive	Weak	1	66	*Corynebacterium hansenii*	AM946639	99

HoPAT; Horn powder agar translucency.

Results

Within the histopathological findings, a large number of Gram-positive cocci and bacilli and a few Gram-negative cocci attached to the borders of the hoof wall cavities were identified (Fig. 2a). In addition, Gram-positive cocci and short rod-shaped bacilli of a slightly different range of sizes invaded into and proliferated inside the horn tissues (Fig. 2b). No structures of hyphae or yeasts were observed in the PAS staining. Thus, in order to focus on bacteria alone, we performed culture using blood agar along with a fungicide.

In the culture results, bacteria were only isolated from cultures performed under aerobic conditions. This was guessable because all pathologically observed bacteria existed in the horn area where the cavity was exposed to air. In *Gidoh*, facultative anaerobes probably did not live or proliferate in the lesions. From the cultured bacteria, we isolated five strains (No. 1–5) (Table 1) showing varying degrees of translucency around the colony on the horn powder-mixed agar by means of the HoPAT test. During this procedure, a yellowish colony of one strain (No. 2) presented stronger and wider translucency than the other four strains (Fig. 3). The HZ results showed that each of the five strains produced one to four different molecular-sized enzymes (Table 1). In other words, the keratolytic activity of each bacterium could be visualized as one or more clear white bands in the blue colored background of the horn-combined acrylamide gel stained with CBB in the secondary screening examination (Fig. 4). Strain No. 1 possessed keratolytic enzymes of 18 and 39 kD in the HZ. Strain No. 2, which showed the strongest translucency in the HoPAT test, possessed four keratolytic bands corresponding to 13, 40, 56, and 68 kD in the HZ. The molecular sizes of the keratolytic enzymes of No. 3 and No. 5 were 36 and 66 kD, respectively. Strain No. 4 showed three keratolytic enzymes, the molecular sizes of which were 35, 70, and 80 kD in the HZ. From the results of 16S rRNA gene sequencing analysis and the subsequent BLASTN identification (Table 1), No. 1, which showed 96% identity to the type strain of *Dermabacter hominis* (accession no.: X91034) [23], could be assigned to the genus level of *Dermabacter* or a novel bacterium. No. 2 was identified as a type strain of *Brevibacterium luteolum* (accession no.: AJ488509) [26] based on its 100% identity to that strain. No. 3 was assigned to a type strain of *Corynebacterium freneyi* (accession no.: AJ292762) [22], as it showed 99% identity to that strain. No. 4, which showed 98% identity to the type strain of *Agrococcus casei* (accession no.: DQ168427) [3], was assigned the genus level of *Agrococcus*. No. 5 was identified as *Corynebacterium hansenii* (accession no.: AM946639) [28], as it showed 99% identity to the type strain of *C. hansenii*.

Discussion

The present study revealed that the hoof wall cavities of the affected horse were the result of Gram-positive aerobic bacteria. Saprophytic infestation of the hoof wall manifested in two different patterns: 1) superficial erosion by bacteria attached to the periphery of cavities and 2) hoof wall collapse with bacteria invading and proliferating in the horn structures. Both manifestations would likely result in bacterium-produced keratolytic enzymes, thereby disrupting the horn.

We were able to screen the keratolytic bacteria from the assorted bacteria existing in a lesion of a *Gidoh*. Five strains showing keratolytic activity were eventually isolated by means of the novel methods of the HoPAT test and HZ as the primary and secondary screening methods, respectively. Based on the 16S rRNA gene sequencing analysis, these bacteria were identified as *B. luteolum, C. freneyi, C. hansenii*, a bacterium assigned to the genus level of *Agrococcus*, and a bacterium assigned to the genus level of *Dermabacter* or a novel bacteria, respectively. Among the bacteria identified in the present study, *B. luteolum* showed the strongest translucency activity in the HoPAT test and produced the most keratolytic enzymes in the HZ. It is suggested that *B. luteolum* might have played a central role in formation of the *Gidoh* but that the other bacteria

also contributed to formation of the *Gidoh* in the studied horse. It is likely that *B. luteolum* can easily disrupt hard horny structures of a horse by its strongly active keratolytic enzymes, thus ultimately contributing to the vulnerability of the horn tissue. The aforementioned hypothesis is supported by a study that found *B. luteolum* to be one of the major bacteria isolated from the damaged laminar layer in chronic laminitis [21]. In addition, in humans, some species of genus *Brevibacterium* are opportunistic pathogens and have been isolated from clinical samples [27, 28].

Although we could not trace the bacterial infestation routes, the most conceivable source of infestation is the ground because the feet of a horse routinely make contact with the bed materials and/or ground contaminated with soil and slurry containing assorted bacteria. Plural keratolytic bacteria could destroy a nonliving organic product, i.e., the horn of the foot, in a manner similar to their activity in a soil environment, where it is known that countless soil bacteria routinely decompose organic substances derived from dead organisms via various types of enzymes. This process in nature allows the recycling of all nonliving biomass to nutrients [14]. Likewise, it was considered that the five identified keratolytic bacteria from the *Gidoh* cooperatively destroyed the nonliving horn by use of plural keratolytic enzymes. In addition, colonization in horse dermal tissues could be considered as an alternative pathogenic source. Further investigation is required to reveal whether each bacterium isolated in the present study is indigenous to the foot epidermis so as to identify the pathogenic source.

In the present research, we were able to establish unique screening methods to identify keratolytic bacteria responsible for formation of the *Gidoh* using a combination of routine bacteriological analysis, biochemistry, and molecular biotechnology. The double screening procedures of the HoPAT test and HZ were useful and easy techniques for isolating the keratolytic bacteria from the horn lesions. Because the present study was an initial investigation, clarification of what other kinds of bacteria are related to the *Gidoh* is required, along with investigation of the prevalence of keratolytic bacteria infestation.

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