別紙様式第４

学　位　論　文　要　旨

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[論文題名]

Transcellular penetration of *Treponema phagedenis* isolated from papillomatous digital dermatitis in polarized normal human epidermal keratinocytes *in vitro*

(牛趾乳頭腫症由来 *Treponema phagedenis* は *in vitro* *牛赴乳頭腫症由来 Treponema phagedenis* は *in vitro* においてヒト角化上皮細胞を細胞内ルートで貫通する)

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[要　旨]

Papillomatous digital dermatitis (PDD) is a contagious foot disease of dairy cattle and its high prevalence worldwide can be attributed to the spread of modern rearing systems. PDD often leads to lameness due to severe pain, resulting in decreased body weight and milk production, and thereby serious economic loss and animal welfare problems. The presence of multiple *Treponema* species consistently and predominantly in PDD lesions. Therefore, these treponemes are considered to be the most important agents in the pathogenesis of the disease. Moreover, multiple bacteria other than *Treponema* species were also detected in the lesions, suggesting that PDD is a polymicrobial infection. it is considered that these organisms have a propensity for tissue invasion and play an important role in inflammation and lesion formation. However, the pathogenetic role of these organisms has not been fully examined due to their fastidious culture requirements *in vitro*.

T. denticola, which is associated with periodontal disease, has a variety of virulence factors. The organism produces dentilisin, a chymotrypsin-like protease, which is thought to facilitate penetration of the epithelial cell layer by disruption of tight junction proteins. The bacterial translocation pathway *in vitro* has been investigated using polarized epithelial cells combined with measurement of transepithelial electrical resistance (TER) using a membrane insert system to indicate the integrity of tight junctions. Although we have isolated *T. phagedenis* from PDD lesions, its role in pathogenesis–especially the mechanism of tissue penetration–has not been examined. In the present study, we tested whether *T. phagedenis* strains isolated from PDD produce proteolytic enzyme(s) and have the ability to translocate in polarized epidermal keratinocytes *in vitro*.

A total of 10 *T. phagedenis* strains (HT201, YG3903R, CH9, HG42, IZ6-2, HD27-4, HD22R11, HD26R, HD21R-R7 and HD26-67) isolated from PDD lesions of dairy cattle (Holstein) in Japan, *T. phagedenis* ATCC27087 isolated from a human genital organ and *T. denticola* JCM8225 isolated from the human oral cavity were used as control. Detection of proteolytic activity was examined by casein hydrolysis test. One loopful of 10 *T. phagedenis* strains isolated from PDD lesions and controls was inoculated onto a modified skim milk agar (mSMA) plates, and then incubated at 37°C for 7-10 days under anaerobic conditions. Proteolytic activity was detected as a transparent zone around the inoculated bacterial cells. Analysis of epithelial barrier integrity was determined by polarized normal human epidermal keratinocytes (NHEKs) monolayers using a membrane insert system. Transepithelial electrical resistance (TER) was monitored the effect of *Treponema* spp. to disrupt the integrity to NHEK cell which inoculated individually, and co-inoculation. The migrating pathway and internalized *T. phagedenis* in NHEK cells were observed by immunofluorescence procedures. The evidence of cell disruption by treponemes was determined by immunofluorescence staining of tight junction protein ZO-1.

After incubation for 7–10 days under anaerobic conditions, *T. denticola* JCM8225 showed considerable proteolytic activity and formed distinct transparent zones around bacterial cells inoculated on mSMA plates. However, no transparent zone was observed among any of the PDD-derived *T. phagedenis* strains examined, or the type strain. These results suggested that *T. phagedenis* strains do not possess proteolytic enzyme(s) and/or exploit the proteases produced by other co-infecting bacteria when they invade dermal tissue.

TER across polarized monolayers of the NHEK cells was not affected by either of the PDD strains of *T. phagedenis* examined, as well as the type strain, during the experimental period. In contrast, TER decreased significantly ($P<0.05$) to 76.8 ± 4.2% and 73.0 ± 5.5% at 24 and 48 hr after inoculation of *T. denticola* JCM8225, respectively. Migration of treponemes from the apical side to the basolateral side was confirmed at 6, 12, 24 and 48 hr after inoculation by microscopic observation and by *Treponema*-specific PCR using the basolateral medium. PCR amplicons of *T. phagedenis* strains HT201 and ATCC27087 were detected within 12 hr after cell inoculation, whereas *T. phagedenis* strain YG3903R was detected after 24 hr of the inoculation. The amplicons of *T. denticola* JCM8225 were detected from the basolateral medium during 6-48 hr after inoculation.

The monolayers were infected with *T. phagedenis* PDD isolated strain for 24 hr to investigate the internalization of *T. phagedenis* in NHEK cells, and cell internalization was visualized using a fluorescence microscope. The presence of *T. phagedenis* inoculated in NHEK cells was observed. To further confirm the translocation route of *T. phagedenis* in the NHEK cells, the tight junction protein ZO-1 was subject to immunofluorescence staining after penetration of inoculated bacteria. When *T. phagedenis* YG3903R was inoculated, ZO-1 proteins surrounding NHEK cells were stained strongly, as was the case for the control without bacterial inoculation. In contrast, NHEK cells inoculated with *T. denticola* JCM 8225 showed decreased intensity of ZO-1, suggesting disruption of ZO-1 protein at the tight junction.

Although single inoculation of the live *T. phagedenis* strain examined did not change the TER values during the experimental period, inoculation of *T. phagedenis* together with live *T. denticola* resulted in a significant decrease ($P<0.05$) of TER after 48 hr of incubation, as well as after single inoculation of live *T. denticola.* However, the TER values did not vary according to the combination of live *T. phagedenis* and heat-killed *T. denticola* during the experimental period, as was seen for single inoculation of live *T. phagedenis*. Furthermore, counting of fluorescence-labeled *T. phagedenis* cells demonstrated.

These findings may explain why multiple treponemes exist in the same PDD lesion. If such a bacterial community is able to compensate for the lack of pathogenicity of bacteria present in PDD lesions, it would confer a survival advantage on the bacteria, thus causing illness and/or sustaining the infection. These present findings may provide a new insight for better understanding the pathogenetic mechanism of polymicrobial infections. However, since a variety of bacteria exist in the lesion and may be associated with PDD pathogenesis, the pathophysiological mechanism of the disease may be complex and difficult to elucidate. To better understand the pathology of PDD as a polymicrobial infection, further approaches will be required. These observations demonstrate that *T. phagedenis* strains isolated from PDD lesions have the potential to pass through dermal tissue transcellularly *in vitro*. Furthermore, co-infection with *T. denticola* producing proteolytic activity accelerated the penetration of *T. phagedenis* across dermal epithelial cells. The present findings may help to explain how *T. phagedenis* is able to reach the deeper tissue in PDD lesions.

備考　論文要旨は、和文にあっては 2，000 字程度、英文にあっては 1，200 語程度