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The microbial synergy of *Peptostreptococcus micros* and *Prevotella intermedia* in a murine abscess model

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This study characterized the microbial interaction of *Peptostreptococcus micros* and *Prevotella intermedia*, the major pathogens of dentoalveolar infection, using a murine model. Subcutaneous injection of *P. micros* cells in the dorsum of the mouse together with living cells of *P. intermedia* resulted in a significantly larger abscess when compared with single injection of the organisms ($P < 0.02$). The abscess size was also significantly increased ($P < 0.05$) when the plate-cultured cell suspension of *P. micros* was injected into mouse with the culture filtrate of *P. intermedia*. The heat-treated culture filtrate of *P. intermedia* also enhanced the virulence of *P. micros*. *P. micros* culture filtrate did not affect the virulence of *P. intermedia*. Interestingly, the virulence of *P. micros* appeared to be enhanced even when the culture filtrate of *P. intermedia* was injected at separate sites in the mouse. These results suggest that a heat-stable product or products of *P. intermedia* increase the virulence of *P. micros* indirectly by altering the host condition, whereas living cells of *P. micros* can directly enhance virulence of *P. intermedia*.

Key words: abscess; dentoalveolar infection; *Peptostreptococcus micros*; *Prevotella intermedia*; synergy

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Although dental health in developed countries is improving, patients with dentoalveolar infection are still frequently encountered. The majority of such infections are related to necrosis of dental pulp tissue (8). However, periodontal diseases, trauma and infection of surgical wounds can also be associated with dentoalveolar infection (2, 8). Although the exact etiology of dentoalveolar infection remains unclear, infection usually involves bacteria residing in the oral cavity (2, 8, 10, 12–14, 17) and is polymicrobial (10, 14, 17). It has also been suggested that microbial interaction plays an important role on the development of the infection (2, 11, 17). In dentoalveolar infection, the bacteria do not occur randomly but are found in specific combinations (10, 16). It has been reported that *Peptostreptococcus* and *Prevotella* are found significantly more frequently than other groups of bacterial species in dentoalveolar infection of endodontic origin (6, 16, 18). Our microbiological investigations have revealed that *Peptostreptococcus micros* and *Prevotella intermedia* are the predominant isolates from acute odontogenic infections (12, 13) and a combination of these species is often encountered in infection (10). From these clinical findings a hypothesis can be investigated to determine whether microbial interaction between *P. micros* and *P. intermedia* contributes to development of purulent infection.

Animal models are widely used to assess microbial pathogenicity in medical research and have been applied to investigations designed for the study of dental infection (4).

The aim of this study was to evaluate bacterial virulence in mono- and mixed infection with *P. micros* and *P. intermedia*, and to clarify the nature of the microbial interaction of these bacteria using a murine abscess model.

Material and methods

Bacteria

P. micros K20 and *P. intermedia* K172 were used in this study. These strains were isolated at Kanazawa University Hospital from pus specimens of patients with dentoalveolar infection. The strains were identified using both biochemical and genetic methods using Rap ID ANA II (Innovative Diagnostic System, Norcross, GA) (12, 13) and MicroSeq 500 16S rDNA Bacterial Sequencing

Kit (PE Applied Biosystems, Foster, CA).

Animal model

The virulence of bacteria was determined using a murine subcutaneous abscess model that has been described previously (4). Six-week-old female ICR mice (Kiwa, Wakayama, Japan) raised under standard conditions were employed in this study. Ten mice per experimental group were used in all investigations.

Preparation of the inocula

The study used combinations of plate-cultured cell suspensions, broth cultures or culture filtrates as inocula. Bacterial concentrations of each inoculum (colony forming units per ml; cfu/ml) were determined using serial 10-fold dilutions, as described previously (11).

Plate-cultured cell suspension

P. micros and *P. intermedia* were cultured on Brucella HK agar (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) with 5% sheep blood in an anaerobic atmosphere at 37°C for 48 and 24 h, respectively. Resulting bacterial colonies were suspended in fresh Schaedler broth (Becton Dickinson, Cockeysville, MD) at a concentration of 2×10^9 to 4×10^{10} cfu/ml.

Broth culture and culture filtrate

P. micros and *P. intermedia* were cultured in the Schaedler broth with 5% sheep blood in an anaerobic atmosphere at 37°C for 48 and 18 h, respectively. Resulting growth from these broth cultures was estimated at 4×10^9 to 7×10^9 cfu/ml and 1×10^9 to 3×10^9 cfu/ml for *P. micros* and *P. intermedia*, respectively. The broths were centrifuged at 1200 *g* for 10 min and the bacterial cells removed by filtration using a 0.22 μm pore size membrane filter (Millipore, Bedford, MA). Heat-treated culture filtrate was prepared by autoclaving the filtrate at 121°C for 20 min.

Experimental design

Virulence of *P. micros* and *P. intermedia* in mono- and mixed infection. A 100-μl volume of the plate-cultured cell suspension of each strain was injected subcutaneously in the dorsum of the mouse to evaluate virulence of the bacteria in mono-infection. In order to determine bacterial virulence of mixed infection, the plate-cultured cell suspension (50 μl) of *P. micros* was mixed with an equal volume of the plate-cultured cell suspension of *P. intermedia* and this preparation (100 μl) was injected into the mouse. The broth culture of these strains was also injected into the mice in a similar manner.

Effect of co-injection of bacteria and filtrates on pathogenicity in the mouse

A 50-μl volume of the plate-cultured cell suspension of one strain mixed with an equal volume of broth culture, culture filtrate or heated-treated culture filtrate of the other strain was injected into the dorsum of the mouse. As the control, 50-μl of the plate-cultured cell suspension of the strain was injected with 50 μl of uncultured sterile Schaedler broth.

Effect of co-injection of bacteria and filtrates (at separate sites) on pathogenicity in the mouse

A 50-μl volume of plate-cultured cell suspension of one strain was injected into the dorsum of the mouse. Concurrently, 50 μl of culture filtrate or the heat-treated culture filtrate of the alternate strain was injected intra-peritoneally. A 50-μl volume of uncultured sterile Schaedler broth was used as the control.

Assessment of virulence

The injected mice were monitored at 12-h intervals for evidence of infection. Seven days after injection, the size of the abscess in each mouse was measured. This involved measuring the abscess size in the area of the localized suppurative lesion. Surrounding areas of inflammation, as indicated by edema and redness, were excluded from the measurement. The pus from the abscess was examined microbiologically to confirm the presence of inoculated bacteria and the absence of other bacterial contamination, as described previously (11).

Statistical analysis

Statistical comparisons of size of the abscess were performed by the Student's *t*-test.

Results

Virulence of *P. micros* and *P. intermedia* in mono- and mixed infection

The pure plate-cultured cells of bacteria resulted in abscess development. It was interesting that the abscesses produced by 1×10^{10} cfu/ml of *P. intermedia* were significantly larger than those produced by 3×10^{10} cfu/ml of *P. micros* ($P < 0.001$, Fig. 1).

When the plate-cultured cells of *P. micros* and *P. intermedia* were co-injected

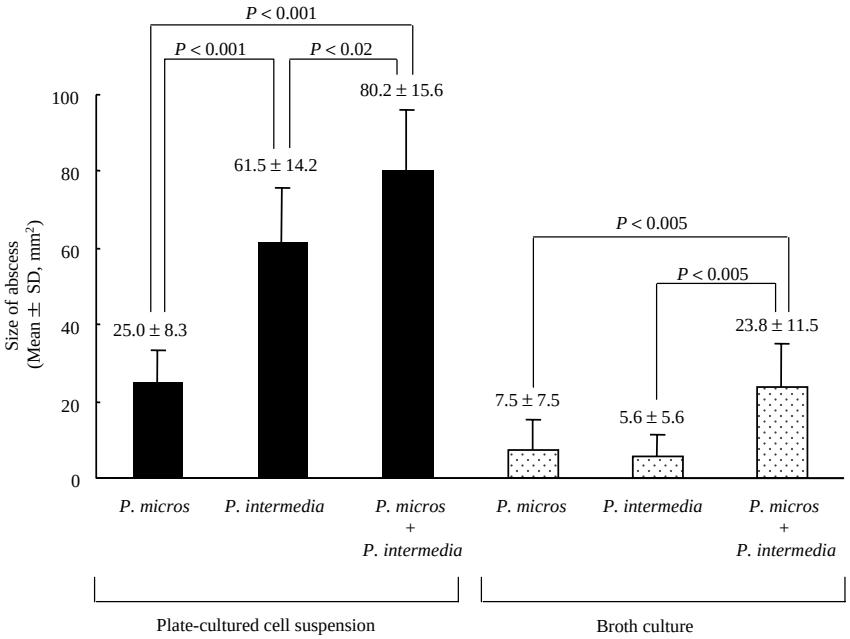

Fig. 1. Virulence of *P. micros* and *P. intermedia* in mono- and mixed infections. Bacterial concentration of inocula: *P. micros* plate-cultured cell suspension, 3×10^{10} cfu/ml; *P. intermedia* plate-cultured cell suspension, 1×10^{10} cfu/ml; *P. micros* broth culture, 7×10^9 cfu/ml; *P. intermedia* broth culture, 3×10^9 cfu/ml.

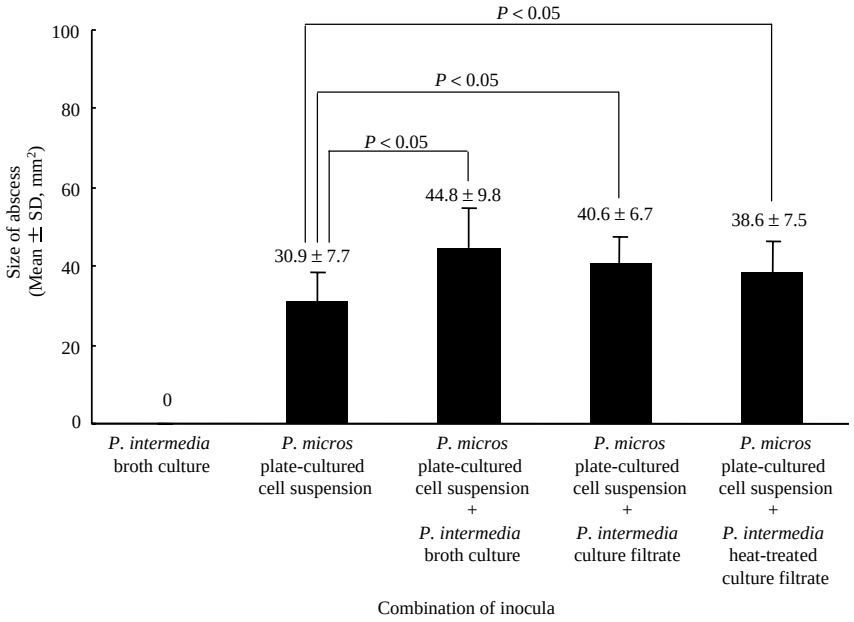

Fig. 2. Effect of *P. intermedia* culture filtrate on virulence of *P. micros* when co-injected into the mouse (the dorsum). Bacterial concentration of inocula: *P. micros* plate-cultured cell suspension, 4×10^{10} cfu/ml; *P. intermedia* broth culture, 1×10^{9} cfu/ml.

into the mouse, the size of the abscess significantly increased compared to mono-infection ($P < 0.02$, Fig. 1). Similarly, mixtures of the broth cultures of these strains produced significantly larger abscesses than the pure broth cultures ($P < 0.005$, Fig. 1).

Effect of *P. intermedia* culture filtrate on virulence of *P. micros* when co-injected into the mouse

Neither *P. micros* nor *P. intermedia* culture filtrate was capable of inducing any lesions in the mice. Injection of *P. micros* plate-cultured cell suspension mixed with the culture filtrate of *P. intermedia* resulted in a $40.6 \pm 6.7\,mm^2$ abscess, which was significantly larger than that of the control ($P < 0.05$, Fig. 2). Abscesses produced by mixtures of plate-cultured cell suspension of *P. micros* and the heat-treated culture filtrate of *P. intermedia* were also significantly larger than those of controls ($P < 0.05$). There was no statistical difference in the size of the abscess between the heat-treated and non-heat-treated culture filtrates.

Effect of *P. micros* culture filtrate on virulence of *P. intermedia* when co-injected into the mouse

When a mixture of the plate-cultured cell suspension of *P. intermedia* and culture filtrate of *P. micros* was injected into the dorsum of mouse, the size of the abscess did not differ statistically from that of the control (Fig. 3). However, the plate-cultured cell suspension of *P. intermedia* mixed with the broth culture of *P. micros* produced significantly larger abscesses than the control ($P < 0.01$).

Effect of *P. intermedia* culture filtrate on virulence of *P. micros* when injected at separate sites of the mouse

When the plate-cultured cell suspension of *P. micros* was injected into the dorsum and the culture filtrate of *P. intermedia* was injected into the abdomen of the same mouse, the abscesses produced were significantly larger than those of the controls ($P < 0.001$, Fig. 4). Furthermore, the size of the abscess increased significantly ($P < 0.001$) when plate-cultured cell suspension of *P. micros* was injected into the dorsum of the mouse together with intraperitoneal injection of *P. intermedia* heat-treated culture filtrate.

Effect of *P. micros* culture filtrate on virulence of *P. intermedia* when injected at separate sites of the mouse

When plate-cultured cell suspensions of *P. intermedia* were injected into the dorsum of the mouse at the same time as intraperitoneal injection of the *P. micros* culture filtrate (or heat-treated culture filtrate) there was no significant difference in abscess size compared with controls (Fig. 5).

Discussion

It is generally accepted that bacteria producing large abscesses in animal models can be regarded as having significant pathogenic potential. In the present study, abscesses produced by 1×10^{10} cfu/ml of *P. intermedia* were significantly larger than those produced by 3×10^{10} cfu/ml of *P. micros* ($P < 0.001$, Fig. 1). Strains of *P. micros* require a greater number of living cells to induce abscess formation compared with *P. intermedia,* as shown

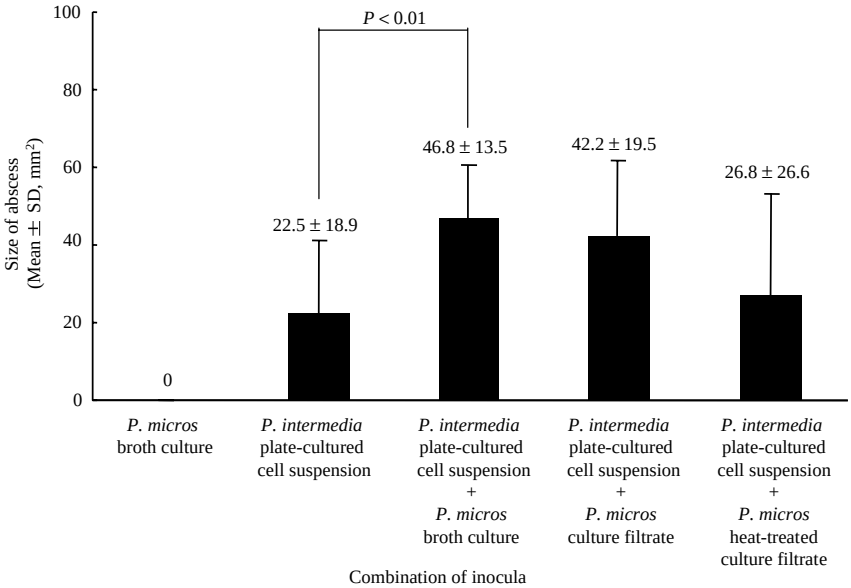

Fig. 3. Effect of *P. micros* culture filtrate on virulence of *P. intermedia* when co-injected into the mouse (the dorsum). Bacterial concentration of inocula: *P. micros* broth culture, 4×10^{9} cfu/ml; *P. intermedia* plate-cultured cell suspension, 2×10^{9} cfu/ml.

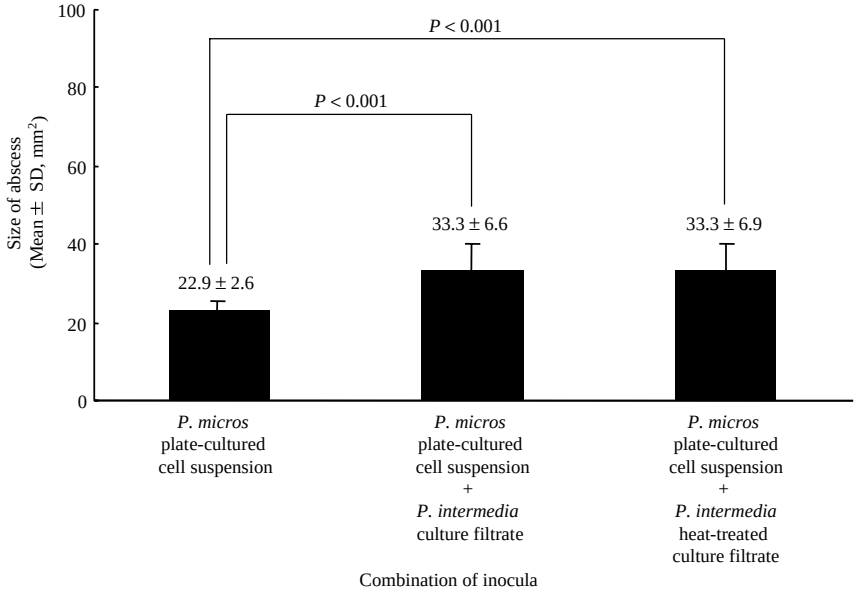

Fig. 4. Effect of *P. intermedia* culture filtrate on virulence of *P. micros* when injected at separate sites of the mouse (the dorsum and the abdomen). *P. micros* plate-cultured cell suspension (3×10^{10} cfu/ml) was injected to the dorsum in the mouse. At the same time, culture filtrate, heat-treated culture filtrate of *P. intermedia* or an uncultured sterile Shaedler broth was injected into the abdomen in the same mouse.

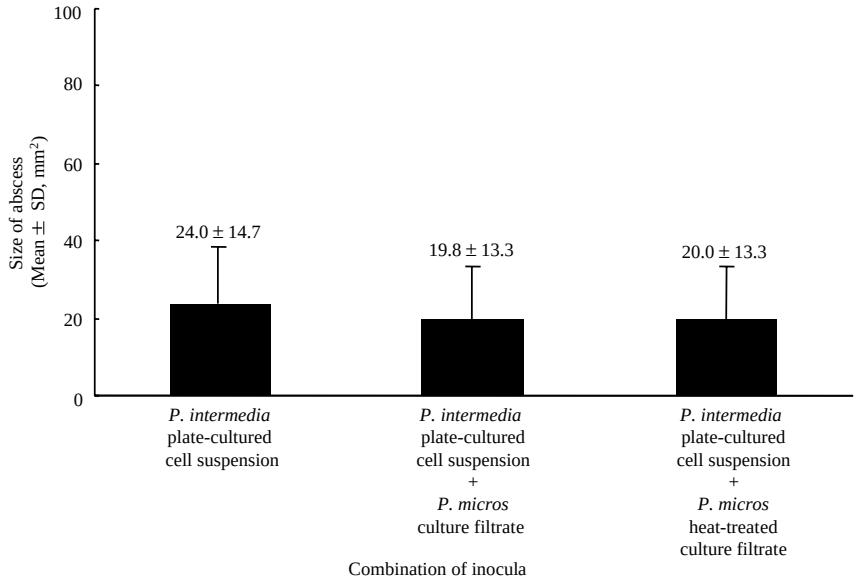

Fig. 5. Effect of culture filtrate of *P. micros* on virulence of *P. intermedia* when injected at separate sites of same mouse (the dorsum and the abdomen). *P. intermedia* plate-cultured cell suspension (2×10^{9} cfu/ml) was injected to the dorsum in the mouse. At the same time, culture filtrate, heat-treated culture filtrate of *P. micros* or an uncultured sterile Shaedler broth was injected into the abdomen in the same mouse.

previously in a separate animal model (9). *P. intermedia* may have a greater ability to induce purulent infection than *P. micros*, although the pathogenic potential varies between strains.

A combination of *P. micros* and *P. intermedia* is frequently encountered in human dentoalveolar infections (6, 16, 18) and may be indicative of a bacterial interaction between these organisms that could play an important role in the development of purulent dental infections. In the present study, mixed infection of *P. micros* and *P. intermedia* produced significantly larger abscesses than mono-infection of either organism ($P < 0.02$, Fig. 1). We confirmed that other *P. micros* and *P. intermedia* strains also exhibited this synergistic behavior when combined in mixed infection using this animal model (data not shown). Such findings of microbial synergy are in agreement with those reported by Brook et al. (1) and van Dalen et al. (3).

When *P. micros* plate-cultured cell suspension was mixed with the culture filtrate of *P. intermedia* and injected into the dorsum of the mouse, the virulence of *P. micros* was apparently increased (Fig. 2). Enhancement of *P. micros* virulence was also found when *P. micros* plate-cultured cell suspension was injected with the heat-treated culture filtrate of *P. intermedia*. It is highly likely that product (s) of *P. intermedia*, probably a heat-stable substance (s) serves to enhance the virulence of *P. micros*. On the other hand, the culture filtrate of *P. micros* did not affect the virulence of *P. intermedia* (Fig. 3). It is worth noting, however, that the broth culture of *P. micros* enhanced the virulence of *P. intermedia* (Fig. 3). It appears that it is the living cells of *P. micros*, and not its products, that are important in enhancing the virulence of *P. intermedia*.

The following theories are suggested as mechanisms of microbial synergy in a mixed infection:

- the combination of microbes offers enhanced protection to host defenses;
- essential nutrients for one component of the relationship are provided by the other;
- alteration of the local environment occurs which increases the virulence of one (or more) of the bacterial components (15).

In the present study, when the plate-cultured cell suspension of *P. micros* was injected with the culture filtrate of *P. intermedia* at separate sites of the mouse, virulence of *P. micros* was enhanced (Fig. 4). Enhancement of *P. micros* virulence was also observed when heat-treated culture filtrate of *P. intermedia* was injected (Fig. 4). It may be possible that the culture filtrate of *P. intermedia* affects the host's condition and subsequently enhances the virulence of *P. micros*. It has been reported that short-chain fatty acids produced by *P. intermedia* suppress human polymorphonuclear neutrophil functions (19). Moreover, the lipopolysaccharide of black-pigmented strictly anaerobic Gram-negative bacilli suppresses the complement action of host (7). *P. intermedia* is a member of the black-pigmented Gram-negative bacilli (5) and could theoretically affect host defense mechanisms in this manner. Heat-stable fatty acids and lipopolysaccharide may have been associated with the enhancement of *P. micros*

virulence observed in the current study. However, further investigation is required to characterize any heat-stable substances generated.

In contrast to *P. intermedia*, culture filtrates of *P. micros* injected into the abdomen did not affect the virulence of *P. intermedia* (Fig. 5). This finding suggests that there is no equivalent product of *P. micros* that contributes in a similar fashion to *P. intermedia* virulence.

The synergistic nature observed in this study is similar to that of *Streptococcus constellatus* and *Fusobacterium nucleatum*. *S. constellatus* has been reported to enhance the virulence of *F. nucleatum* through interaction between living cells, while *F. nucleatum* affects host defenses through its products, and indirectly enhances virulence of *S. constellatus* (11). *S. constellatus* and *F. nucleatum* are often isolated from dentoalveolar infection (12, 13). The nature of microbial synergy in dentoalveolar infection has been partly determined. However, further study is necessary to address the microbial interaction induced by other combinations of bacteria and to clarify the etiology of dentoalveolar infection.

In conclusion, *P. micros* and *P. intermedia* enhance virulence by different mechanisms in mixed infection. Furthermore, the synergy of these organisms could play a contributory role in the occurrence and prognosis of dentoalveolar infection.

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