Isolation of *Porphyromonas gingivalis* from esophageal squamous cell carcinoma tissues using fastidious anaerobe agar

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Abstract:

To isolate and culture *P. gingivalis* from esophageal squamous cell carcinoma (ESCC) tissues using fastidious anaerobe agar (FAA). The effect of FAA was evaluated by assessing the growth cycle and colony characteristics of the reference strain Pg ATCC 33277. Positive *P. gingivalis* status was confirmed by qPCR. Tissue samples were homogenized and cultured through continuous streaking on the FAA plate in an anaerobic chamber. Preliminary identification of isolates was conducted using Real time PCR and 16S rRNA sequencing . FAA exhibited higher sensitivity and better suitability for the growth of *P. gingivalis* compared to Brain Heart Infusion Agar (BHIA) and Tryptic Soy Agar (TSA). One *P. gingivalis* strain (designated LyEC01) was successfully isolated from tissue of a patient with ESCC in central China. Using FAA in an anaerobic chamber, agar colonies and cultures of LyEC01 was obtained and purified. In addition, the strain was further analyzed through Sanger sequencing. LyEC01 is a novel clinical strain of *P. gingivalis*, marking the first successful iso-

Isolation from ESCC tissue using the FAA method.

Key words: esophageal carcinoma; *Porphyromonas gingivalis*; isolation; fastidious anaerobe agar

Introduction

Porphyromonas gingivalis (*P. gingivalis*) is an obligate anaerobic, Gram-negative common oral bacterium in the etiopathogenesis of periodontitis and other systemic diseases[1,2]. However, accumulating evidence demonstrates that *P. gingivalis* could be enriched in upper gastrointestinal tract tumors, particularly in esophageal squamous cell carcinoma (ESCC), and closely related to the progression and metastasis of ESCC[3-5]. The abundance of *P. gingivalis* in tumor tissues and IgG in serum suggests its potential clinical application as a biomarker for the diagnosis, prognosis and treatment of ESCC[6,7]. *P. gingivalis* expresses several virulence factors that enable it to colonize, invade, and disrupt epithelial tissues[8,9]. The severity of *P. gingivalis* infections differs across strains due to their inherent variability, which could potentially change its role as a keystone pathogen[10-12]. Previous studies used methods such as polymerase chain reaction (PCR), *in situ* RNA hybridization, IHC, and sequencing to detect *P. gingivalis* and determine its presence in clinical samples[13-15], but did not isolate and culture *P. gingivalis* from clinical samples, especially tissues, perhaps due to the absence of reliable selective methods.

Isolation and identification of bacteria from clinical sample is conventional yet highly reliable methods. *P. gingivalis* isolates were cultured on the brucella agar (BA), Brain Heart Infusion Agar (BHIA), Trypticase soy agar (TSA), all supplemented with defibrated horse or sheep blood, hemin, and menadione[16-19]. Fastidious Anaerobe Agar (FAA) supports excellent growth of most clinically anaerobes due to its enriched peptones, which enhances the growth organisms like, *Fusobacterium* spp[20]. Moreover, FAA is commercially available from multiple producers, making it a viable candidate for isolation of *P. gingivalis* from clinical samples. The aim of this study was to use conventional cultural methods with FAA to isolate and identify *P. gingivalis* from tumor tissues, and consequently, to facilitate the investigation of *P. gingivalis*' genotypic diversity.

Here, we demonstrated that the use of FAA as an alternate method for efficient and reliable isolation of *P. gingivalis* from human tissue samples.

Materials and methods

Sample collection and preparation.

Patients diagnosed with histologically confirmed primary tumors were recruited for this study, including 3 patients of ESCC at the First Affiliated Hospital of Henan University of Science and Technology during March to May in 2022. The fresh tumor tissues were taken from primary surgical resection, and then parts of them were washed by cold PBS at least twice, homogenized and resuspended in 200 μL of pre-reduced tryptic soy broth (TSB)[21]. Some portions were prepared for DNA extraction, and the rest of tissues were frozen at −80 ℃ until further procession. All participants provide written informed consent prior to samples collection. The study was approved by the Ethics Committee under no. 2022-03-B110.

Cultivation of *P. gingivalis* ATCC 33277

Take ATCC 33277 strain in the exponential growth phase, adjust the bacterial suspension concentration to 10^9 CFU·mL^{-1}, and perform serial dilutions to 10^6 CFU·mL^{-1}, 10^5 CFU·mL^{-1}, 10^4 CFU·mL^{-1}, and 10^3 CFU·mL^{-1}. Spread an appropriate amount onto FAA, BHIA, and TSA blood agar plates. Place them into an anaerobic incubator (85% N_2, 10% H_2, 5% CO_2) at 37 ℃ for 5 to 8 days.

Isolation and purification of *P. gingivalis* clinical strain

The homogenized tissue samples were diluted and plated onto pre-reduced FAA plate supplemented with 5% defibrated sheep blood, 5 μg/mL hemin-menadione, by continuous scribing. The plates were incubated in duplicate under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37 ℃ for 7 − 14 days. The bacteria grown were selected on the basis of size, color, shape, and staining[18]. The colonies with black-

pigmented and shape of rod were identified by direct-PCR and Gram staining. The gram-negative rods that tested positive for amplification in direct-PCR were considered as *P. gingivalis*. Repeat the process of selecting colonies, identification, plating, and incubation for a minimum of two rounds to ensure the acquisition of purified colonies.

Enrichment cultures

The picked single *P. gingivalis* black colony was cultured in 500 μL pre-reduced TSB for 24 – 48 h in anaerobic chamber. Supplement with fresh pre-reduced TSB up to a volume of 5 mL when the culture reaches turbidity. After 48 h of growth in an anaerobic chamber (37 ℃), 5 mL of the cultures was further expanded to 50 mL. After preliminary identification by Gram staining and direct-PCR, proceed with full-length amplification of the 16S rRNA and extraction of genomic DNA.

Blood agar plates and all other media were pre-reduced in the anaerobic chamber for at least 24 h.

DNA extraction, polymerase chain reaction

Genomic DNAs from esophageal homogenate, and subcultures obtained from single purified colonies that had been grown in TSB for 24 – 48 h in anaerobic chamber were extracted by the Micro Elute Genomic DNA Kit (# D3096 – 02, Omega Bio-Tek, Inc. Norcross, Georgia., USA), and eluted in 50 μL of preheated Elution Buffer, and then detected by real-time PCR or genomic sequencing.

The absence of *P. gingivalis* in plaque samples, picked colonies, and purified isolates were all tested by direct real time PCR without DNA extraction, the procedure was described in a previous study[13].

16S rRNA amplification and Sanger sequencing

Real time PCR and 16S rRNA sequencing were used for preliminary identification. The purified isolates were subjected to amplification with universal bacterial primer pair – 27F/1492R[22], and DNA sequencing in both direction by Azenta® (Suzhou, China). The obtained map results were analyzed by Chromas 2.22 (Technelysium Pty. Ltd.) and sequencing results were analyzed via BLAST search in NCBI (https://blast.ncbi.nlm.nih.gov/). Bacterial species were identified if subjects showed the lowest expectation (E) value in the list of BLAST results.

Results

Cultivation of *P. gingivalis* ATCC 33277 on three different agar plates

FAA, BHIA, and TSA plates were used to evaluate the performance of *P. gingivalis* cultivation and isolation. As shown in Figure 1, when 10^4 CFU·mL^{-1} ATCC 33277 was cultured on three types of solid media—FAA, BHIA, and TSA—distinct ink-black characteristic colonies appeared on the FAA plates by day 6, with uniform size, shape, and color. In contrast, on the BHIA and TSA plates, the colonies turned completely black around days 7 to 8, with fewer numbers than on the FAA plates, and exhibited inconsistent sizes and colors. Additionally, the minimum bacterial concentration required for growth and formation of black colonies was observed to be 10^2 CFU·mL^{-1} on FAA, while it was 10^3 CFU·mL^{-1} on BHIA and TSA (Table 1). The results indicate that compared to TSA and BHIA plates, FAA plates allow for a shorter cultivation period and better sensitivity for *P. gingivalis* growing.

Table 1 The Growth of ATCC 33277 on the FAA, TSA, and BHIA plates

菌液稀释浓度/(CFU·mL)$^{-1}$	48 h			120 h		
	FAA	BHIA	TSA	FAA	BHIA	TSA
1×10^6	+ + +	+	+	+ + +	+ + +	+ + +
1×10^5	+ + +	+	+	+ + +	+ + +	+ + +
1×10^4	+ +	+	+	+ + +	+ +	+ +
1×10^3	+ +	–	–	+ + +	+ +	+
1×10^2	+ +	–	–	+ +	+	+

Isolation of *P. gingivalis* in esophageal tissue using FAA

Tissue samples from patients with ESCC were collected for isolating *P. gingivalis* by FAA. Real-time quantitative PCR (qPCR) was performed on these tissue samples to check for the presence of *P. gingivalis* and its copy number (Fig. 2A). Following PCR, the sample tested positive for amplification of *P. gingivalis* was homogenized and spread-plated onto the culture medium of FAA in an anaerobic condition for 10 – 14 days. As shown in Fig. 2B, several spherical, black

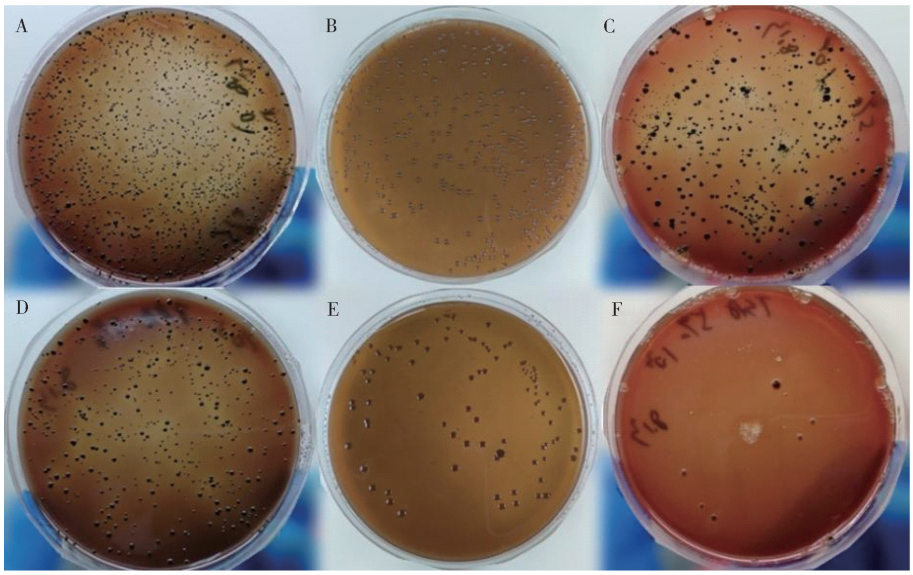

Fig. 1 The Growth Morphology of ATCC 33277 on the plate of FAA, TSA, and BHIA. A ~ C: The cultivation morphology of 10^4 CFU·mL^{-1} ATCC 33277 on FAA, BHIA, and TSA plates under the same duration (6 days); D ~ F: The cultivation morphology of 10^3 CFU·mL^{-1} ATCC 33277 on FAA, BHIA, and TSA plates under the same duration (6 days).

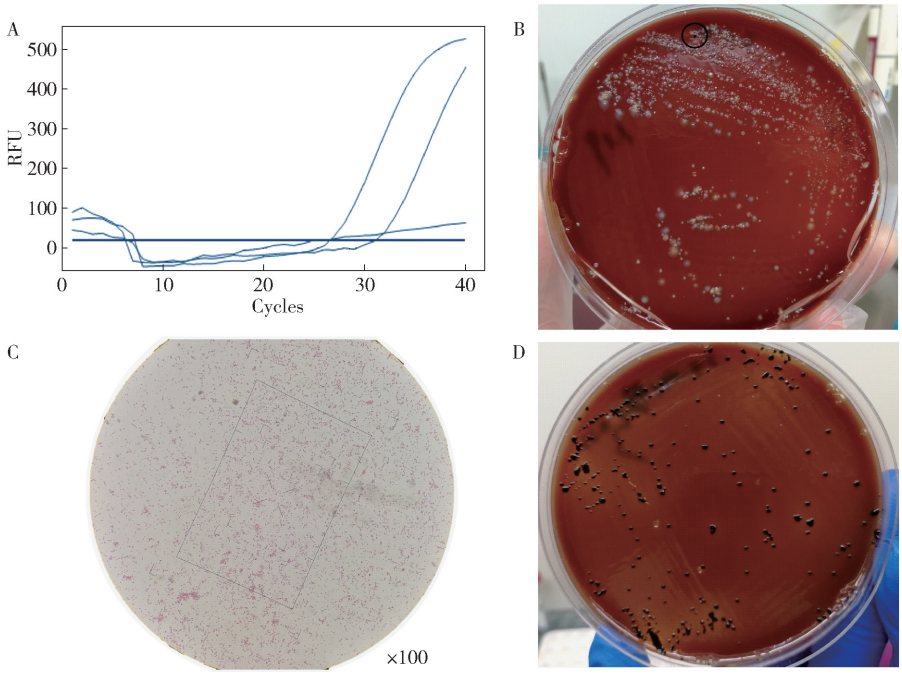

Fig. 2 Isolation of *P. gingivalis* in esophageal tumors using FAA agar. (A) The presence of *P. gingivalis* from tissue samples of ESCC patients was determined by direct-PCR. (B) The colonies were isolated and cultured from tumor tissue of ESCC patient, with FAA. Black circle represents *P. gingivalis* colony. (C) The colony were stained by Gram staining, and Gram-negative bacteria were observed under a microscope (100 ×). (D) The purified spherical, black-pigmented *P. gingivalis* colony.

colonies were seen in the dishes with FAA agar. These colonies were resuspended with pre-reduced TSB, and one part of the bacterial solution was used for Gram staining, while the other part was used for direct-qPCR identification. Among the *P. gingivalis*-positive colonies identified by qPCR, Gram staining results showed that most colonies were pure *P. gingivalis* (Fig. 2C). After three rounds of repeated streaking, purified *P. gingivalis* colonies were obtained, with only a single *P. gingivalis* black clone visible on the plate, with no other bacteria present (Fig. 2D).

Strain Identification and Preservation

Genomic DNA extractedfrom subcultures obtained from single purified colonies was performed 16S rRNA amplification, and sequences were determined by Sanger sequencing. The direct sequencing map was verified on Chromas and the reads were analyzed by BLAST. The map and BLAST analysis confirmed the presence of *P. gingivalis* in the cultures (Fig 3). We named this new *P. gingivalis* strain LyEC01 to indicate that it originates from a case of esophageal cancer tissue in Luoyang.

The strain has been submitted to the General Microorganisms Center of the China General Microbiological Culture Collection Management Committee, and got a preservation number of CGMCC No. 27581.

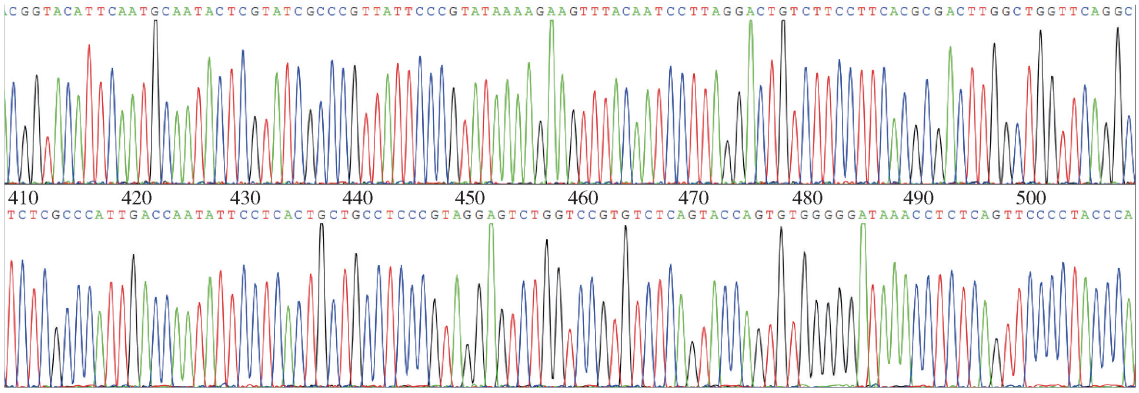

Fig. 3 Sequencing of *Porphyromonas gingivalis* 16S rRNA (partial of sequencing map in forward direction with 27F primer)

Discussion

Bacterial culture played a key role in the association ofspecific bacteria with signs and symptoms of tissue infections. The species and quantities of microorganisms in esophageal tissues are diverse and complex. *P. gingivalis* is considered as a keystone pathogen in human periodontitis[23]. Due to its property of perturbing epithelial tissues and host defense mechanisms, presence of *P. gingivalis* is closely related to the progression of esophageal cancer[5,24,25]. However, isolation and culture of *P. gingivalis* from esophageal tissues remain highly challenging.

In this study, we used FAA blood plates for the direct isolation of *P. gingivalis* from human esophageal tissues. The assay involves two important steps: after PCR verification, the tissue homogenates are immediately streaked onto blood agar plates for inoculation in an anaerobic chamber; and the selective medium used for isolation and purification was FAA plate.

FAA serves as areliable medium for susceptibility testing of fusobacteria and for disk diffusion tests, permitting excellent growth across various manufacturers in different anaerobic incubation systems[20,26]. The use of FAA for the isolation of *P. gingivalis* is not widely reported to date. There are only few studies conducted in the literature comparing different mediums in the detection of *P. gingivalis*, and these studies have been isolated *P. gingivalis* clinical strains from gingival crevicular fluid, saliva, and plaque samples using BA, BHIA or other plates[19,27], but there are no studies that isolate from tumor tissues. We have conducted experiments to evaluate the performance of various agar plates in susceptibility testing for *P. gingivalis*. The results indicated that FAA yielded better endpoints with shorter culture cycle. This is consistent with the results of other studies.

In this study, we successfully isolated and ob-

tained a novel *P. gingivalis* clinical strain from esophageal tumor tissues with FAA plate method. This achievements highlights the potential for isolating an increasing number of *P. gingivalis* strains from human tumor tissues. Such progress may facilitate the investigation of *P. gingivalis*' genotypic diversity, molecular epidemiological studies, and pathogenic mechanism.

Future Directions

Although our study represents a significant step forward in isolation of novel oral bacteria strains, there are limitations that require further investigation. First, to establish LyEC01 as a novel strain of *P. gingivalis* specific to esophageal tumor tissues in the Luoyang, China region, it is essential to repeat these experiments with a larger number of samples. This will ensure reproducibility and confirm the consistency of our findings across multiple cases. Second, deeper genetic analysis of LyEC01 is necessary to identify distinct genomic regions compared to the conventional *P. gingivalis* ATCC 33277 strain. Whole-genome sequencing and comparative analysis could reveal unique sequences or genetic markers that differentiate LyEC01 as a potentially novel strain. Such studies may lead to insights into whether these differences exist at the strain, species, or even genus level. Lastly, functional studies investigating the interactions between *P. gingivalis* and tumor microenvironments would provide deeper understanding of its role in ESCC progression and metastasis. Altogether, addressing these gaps will not only strengthen the case for LyEC01 as a new strain but also contribute to advancing our knowledge of the role of *P. gingivalis* in esophageal squamous cell carcinoma. It is anticipated that such efforts will pave the way for new diagnostic and therapeutic strategies targeting this keystone pathogen.

Competing interests

The authors declare that they have no competing interests in this study.

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