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Original Article

A study of the fluorescence characteristics of common cariogenic microorganisms

Haihua Zhu, Weiwei Lao, Qingguang Chen, Qixia Zhang, Hui Chen

Department of Conservative Dentistry and Periodontics, Affiliated Hospital of Stomatology, College of Medicine, Zhejiang University, China

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Abstract: Objective: To explore the fluorescence characteristics of common cariogenic bacteria: Streptococcus mutans, S. sanguis, Actinomyces viscosus, Prevotella intermedia, Lactobacillus acidophilus, and Candida albicans. Methods: The bacteria were cultured on brain heart infusion (BHI) agar and BHI blood agar, and bacterial colonies were collected for further amplification in liquid medium. Bacterial suspensions in physiological saline were equally divided into three parts for bacteria counting, fluorescence spectrometry detection, and fluorescence microscope examination. Results: The optimal excitation wavelength of the bacteria was 350 nm; their characteristic fluorescence peak position was at 436 ± 4 nm. There was a significant linear correlation between fluorescence intensity and bacterial concentration. The mean optical density (MOD) of S. mutans and L. acidophilus cultivated in BHI blood was significantly higher than that cultivated in BHI agar (110 ± 10 vs. 57 ± 20; 94 ± 16 vs. 31 ± 12, respectively, $P < 0.05$). The MOD of S. sanguis, A. viscosus, and P. intermedia cultivated in BHI blood agar was higher than that cultivated in BHI agar (37 ± 12 vs. 36 ± 11; 43 ± 17 vs. 38 ± 6; 86 ± 21 vs. 72 ± 8, respectively, $P > 0.05$); the opposite was observed for C. albicans. Conclusion: At 350 nm excitation wavelength, 436 ± 4 nm is an indicator for detecting six cariogenic bacteria. The fluorescence energy, Q, is a valuable index reflecting bacterial concentration under fluorescence spectrometry detection. Exogenous fluorescence groups have greater influence on fluorescence intensity and little influence on fluorescence peak position detection.

Keywords: Fluorescence, caries, microorganisms

Introduction

Dental caries is one of the most prevalent diseases in humans; its high incidence and wide distribution severely affect quality of life [1]. At present, primary diagnosis of dental caries involves inspection and probing using dental explorers and dental mirrors which is insufficiently sensitive for detecting initial enamel caries [2]. We hope to discover a new means of diagnosing early tooth decay accurately. One of the key etiological factors of dental caries is cariogenic bacteria, which are research hot spots in oral microbiology [3]. Recent research shows that Streptococcus mutans, S. sanguis, Actinomyces viscosus, Prevotella intermedia, Lactobacillus acidophilus, and Candida albicans are common cariogenic microorganism [4].

Mature plaque produces red autofluorescence when illuminated with blue light [5, 6]. This extrinsic autofluorescence is formed in dental plaque and is found on active caries in particular [7]. Little is known of its exact origin, but it is assumed that red autofluorescence originates from specific bacterial metabolites formed in the oral biofilm, that of gram-negative bacteriain particular, such as protoporphyrin IX [8, 9]. DIAGNOdent instruments are used to diagnose dental caries by detecting the intensity of red autofluorescence from a carious lesion [10]. Actinomyces viscosus, P. intermedia, C. albicans, and Corynebacterium species can produce red autofluorescence in the 600-700 nm wavelength region under blue light, while gram-positive bacteria such as S. mutans, Enter ococcus faecalis, and some Lactobacillus species cannot [7]. Hence, it is important to identify other spectral signatures, especially for detecting gram-positive bacteria. Furthermore, material in the culture medium may also influence fluorescence detection results [11].

Fluorescence spectrometers and fluorescence microscopes are the most common fluorescence analysis instruments and are widely used in quantitative and qualitative cell biology research [12, 13]. Fluorescence spectrometers can be used to determine the fluorescence spectrum of pure substances, and the fluorescence intensity is proportional to the concentration of a substance in its diluent. However, the inner components of live organisms such as bacteria vary greatly. Many fluorescence monomers contribute to bacterial autofluorescence [14]. Therefore, the linear correlations between fluorescence intensity and bacterial concentration still warrant further investigation. Fluorescence microscopes can produce fluorescence images of bacteria, and image analysis software can be used to perform quantitative and qualitative analysis.

The main objectives of this study were to: (i) determine the characteristics or distinctions from the fluorescence spectrum and images for S. mutans, S. sanguis, A. viscosus, P. intermedia, L. acidophilus, and C. albicans; (ii) explore the influence of exogenous fluorescence groups on fluorescence detection; and (iii) provide a foundation for diagnosing early tooth decay.

Materials and methods

Preparation of bacterial samples

Six common species: S. mutans ATCC25175, S. sanguis ATCC10556, A. viscosus ATCC15987, P. intermedia ATCC25611, L. acidophilus ATCC 4356, and C. albicans ATCC9008, were cultured on brain heart infusion (BHI) agar (normal control agar) or BHI agar with 5% sheep blood (BHI blood agar), and then incubated for 48 h at 37°C. Two samplings of each species were obtained from BHI agar and BHI blood agar. Cell suspensions of each bacterium were amplified in liquid medium, and then prepared in phosphate-buffered saline solution (pH 7.4); their concentrations were determined by using DENSIMAT [15, 16]. Each sample was diluted into five concentration gradients: C_0, $C_0/2$, $C_0/4$, $C_0/8$, $C_0/16$, and contained between 10^7 and 10^3 cells/mL in physiological saline prior to fluorescence scanning.

Fluorescence spectroscopy and fluorescence microscopy

Fluorescence spectra were acquired using a Shimadzu RF5301 fluorescence spectrometer (Japan) at 350 nm excitation wavelength. The spectrometer has a single position cell holder for measuring liquid samples. Each bacterial suspension (3 mL) was placed in a high-grade standard (1 cm^2) quartz cuvette (10-mm path length). All measurements were made at room temperature (20-22°C).

Synchronous scanning was used to determine the optimal excitation and emission wavelengths for each bacterium, in which both excitation and emission monochromators were scanned simultaneously with constant wavelength intervals between them [17]. For synchronous spectra, fluorescence emission spectra were recorded with excitation wavelengths of 280 nm, 310 nm, 350 nm, and 390 nm. Each bacterial suspension was scanned twice.

Fluorescence images of each sample were captured using a Nikon Eclipse 80i fluorescence microscope (Japan) for further analysis.

Data processing and image analysis

Fluorescence spectra (sp format) acquired using the spectrometer were exported to GraphPad Prism 5 Demo [18]. Characteristic fluorescence peak position $\lambda em_{1\sim5}$, fluorescence intensity $I_{1\sim5}$, and fluorescence energy $Q_{1\sim5}$ were processed using Excel; monofactor analysis of variance, t-testing, and linear correlation were used for analysis. Fluorescence images were analyzed using Image-Pro Plus 6.0 software [19].

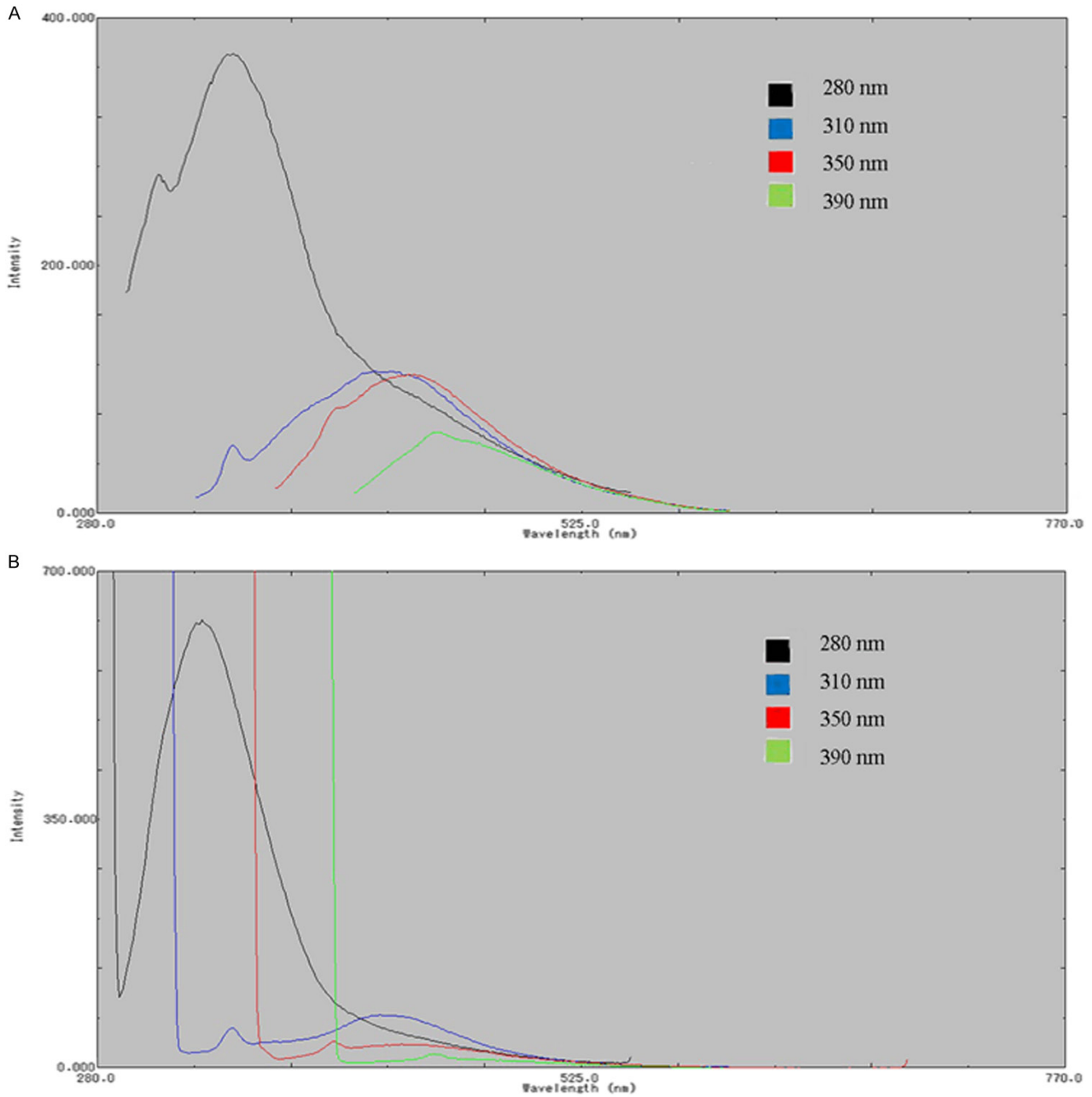
Results

Optimal excitation wavelengths

To determine which excitation wavelength could best detect S. mutans, S. sanguis, A. viscosus, P. intermedia, L. acidophilus, and C. albicans, the fluorescence emission spectra of each bacterium were recorded with excitation wavelengths of 280 nm, 310 nm, 350 nm, and 390 nm. **Figure 1A-F** shows that the longest excitation wavelength for all six species was 280 nm. However, the noise peaks were also highest at this wavelength. In contrast, emission spectra were more stable and undisturbed at 350 nm. We eventually selected 350 nm as the optimal excitation wavelength for all six bacterial species.

Characteristic fluorescence peak positions

At the 350 nm excitation wavelength, the characteristic fluorescence peak position of all six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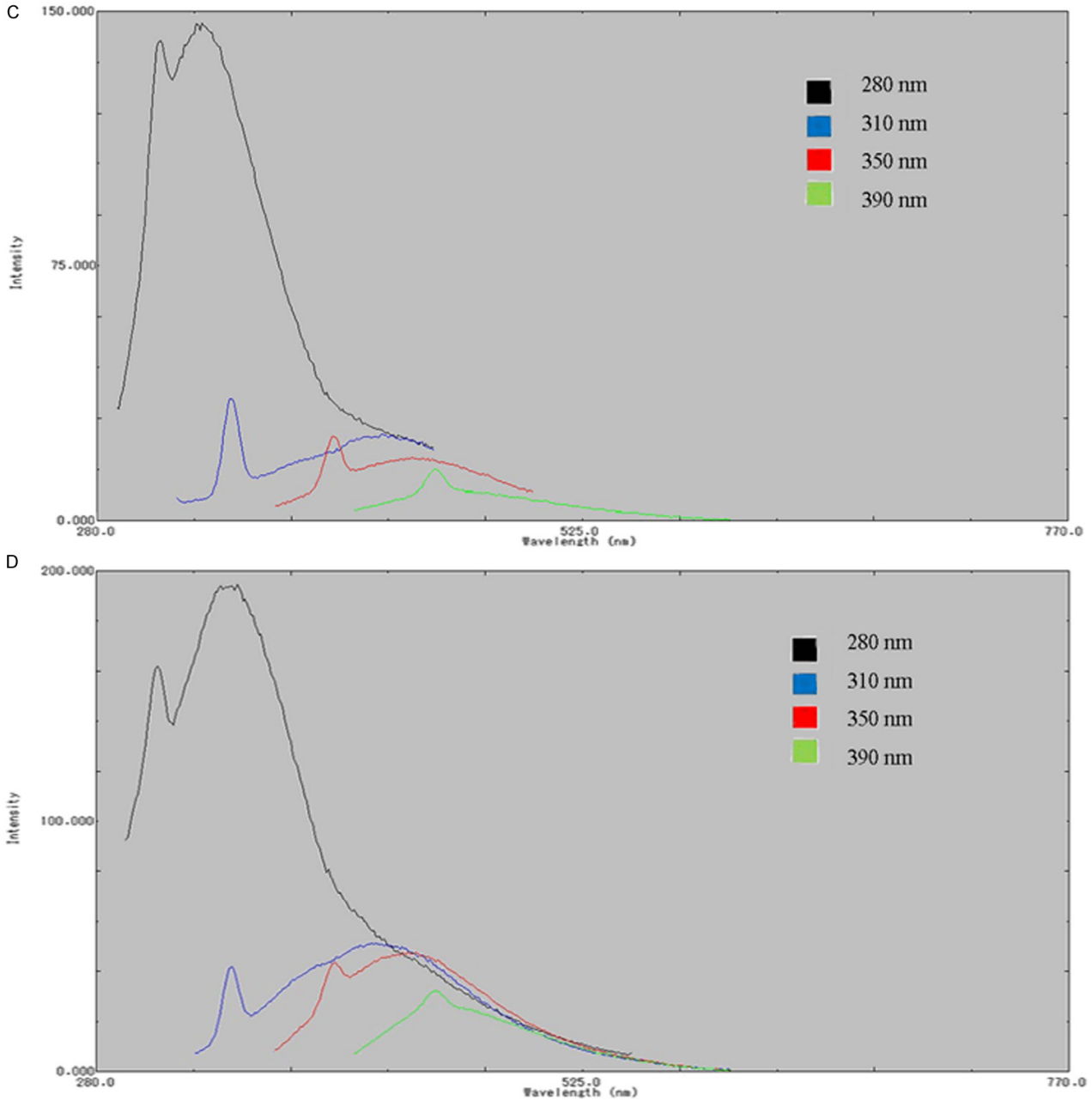
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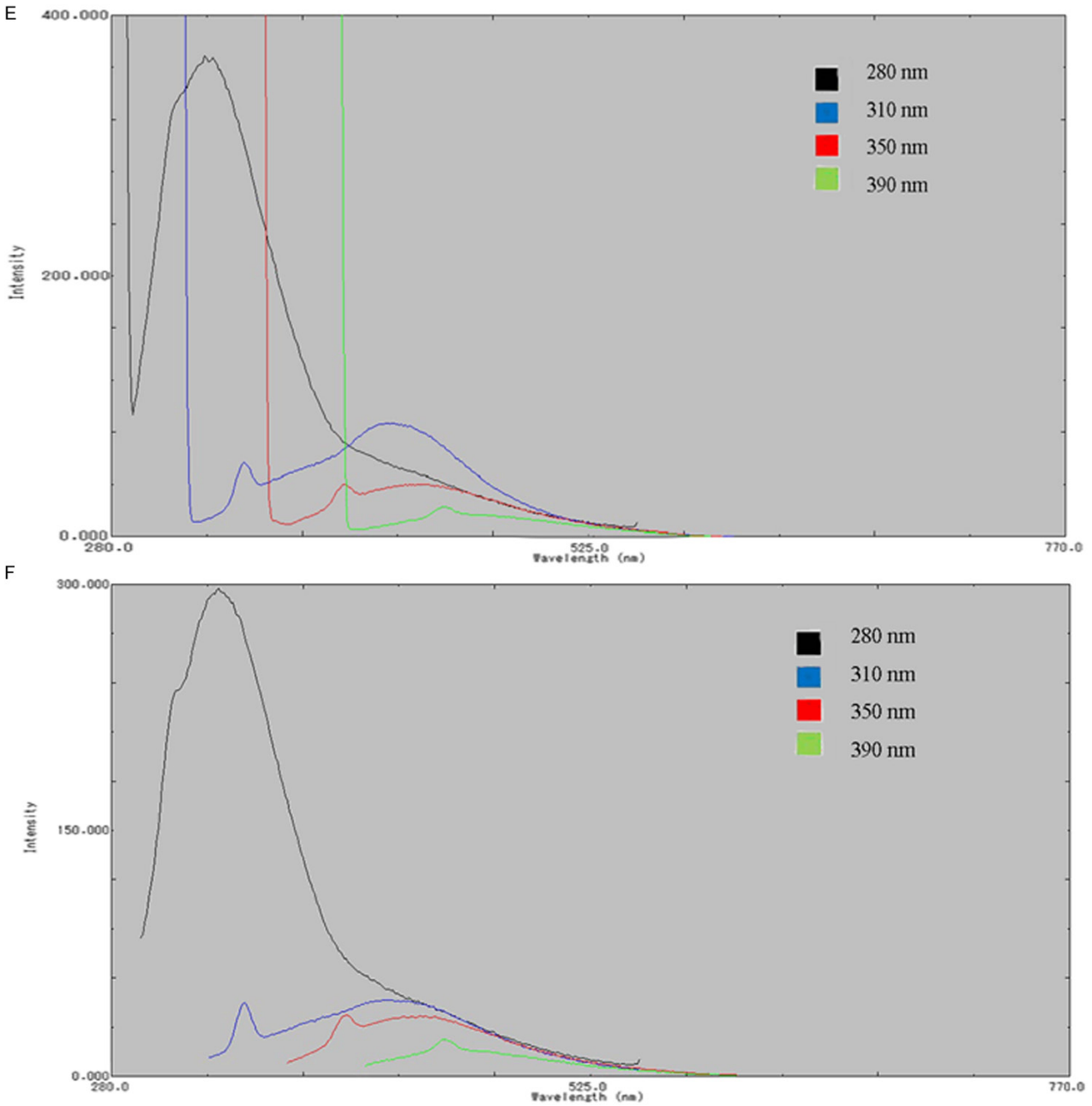
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Figure 1. Emission spectra of bacteria at different excitation wavelengths. (A: S. mutans; B: S. sanguis; C: A. viscosus; D: P. intermedia; E: L. acidophilus; F: C. albicans).

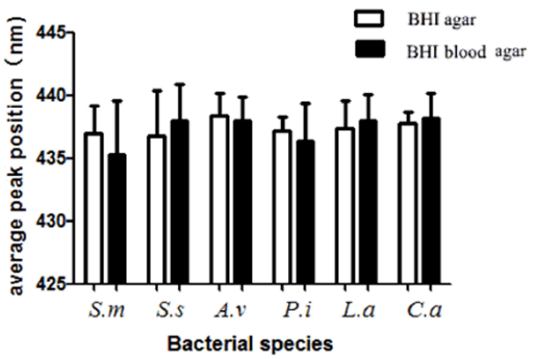

Figure 2. Average fluorescence peak position of bacteria at the 350 nm excitation wavelength. (S.m: S. mutans; S.s: S. sanguis; A.v: A. viscosus; P.i: P. intermedia; L.a: L. acidophilus; C.a: C. albicans).

bacteria, whether cultured on BHI agar or BHI blood agar, was 436 ± 4 nm (**Figure 2**). There was no significant difference between fluorescence peak position values in the same group and between groups (P > 0.05).

Relationship between fluorescence intensity and bacterial concentration

Table 1 presents an overview of the extent of the linear correlation between fluorescence intensity (I) and bacterial concentration (C), or "I-C". The I-C for S. mutans, S. sanguis, A. viscosus, L. acidophilus, and C. albicans was high (R > 0.8). Prevotella intermedia cultured on BHI blood agar had imperfect I-C linear correlation (R = 0.786). We believe that there are fine linear correlations between fluorescence intensity and bacterial concentration, thus bacterial concentrations can be estimated by detecting the fluorescence intensity.

Relative fluorescence energy

The earlier results show that all six bacteria can produce fluorescence in the 370-600 nm wavelength range, and their fluorescence peaks were all in the 432-440 nm range. In our study, $Q_{370\text{-}600\ nm}$ indicates the spectral image area in the 370-600 nm range, and $Q_{432\text{-}440\ nm}$ indicates the spectral image area in the 432-440 nm range. From this, we obtained a relative value from $Q_{432\text{-}440\ nm}/Q_{370\text{-}600\ nm}$, which was not related with bacterial concentration but was influenced by the culture environment and bacterial species.

The $Q_{432\text{-}440\ nm}/Q_{370\text{-}600\ nm}$,of S. mutans, S. sanguis, P. intermedia, and L. acidophilus cultured on BHI blood agar was significantly higher than that cultured on BHI agar (0.068 ± 0.001 vs. 0.062 ± 0.002; 0.079 ± 0.004 vs. 0.067 ± 0.004; 0.073 ± 0.001 vs. 0.070 ± 0.002; 0.081 ± 0.003 vs. 0.072 ± 0.001, respectively; P = 0.001, 0.001, 0.01, 0.002 < 0.05, respectively) (**Table 2**). The $Q_{432\text{-}440\ nm}/Q_{370\text{-}600\ nm}$, of A. viscosus and C. albicans cultured on BHI blood agar did not differ significantly from that cultured on BHI agar (0.069 ± 0.002 vs. 0.071 ± 0.005; 0.067 ± 0.005 vs. 0.070 ± 0.002, respectively; P = 0.40, 0.23 > 0.05, respectively). This shows that exogenous fluorescence groups in the culture environment may improve the fluorescence-producing ability of bacteria. Comparison among groups showed that the $Q_{432\text{-}440\ nm}/Q_{370\text{-}600\ nm}$ of S. mutans cultured on BHI agar was significantly lower than that of S. sanguis, A. viscosus, P. intermedia, L. acidophilus, and C. albicans (0.062 ± 0.002 vs. 0.067 ± 0.004; 0.062 ± 0.002 vs. 0.071 ± 0.005; 0.062 ± 0.002 vs. 0.070 ± 0.002; 0.062 ± 0.002 vs. 0.072 ± 0.001; 0.062 ± 0.002 vs. 0.070 ± 0.002, respectively; P = 0.03, 0.01, 0.001, 0.001, 0.001 < 0.05, respectively). This indicates that S. mutans has relatively weaker fluorescence-producing ability.

Bacterial mean optical density under fluorescence microscopy

The mean optical density (MOD) of S. mutans, S. sanguis, and L. acidophilus cultured on BHI blood agar was significantly higher than that cultured on BHI agar (0.091 ± 0.022 vs. 0.051 ± 0.015; 0.067 ± 0.015 vs. 0.039 ± 0.018; 0.135 ± 0.031 vs. 0.064 ± 0.025, respectively; P = 0.028, 0.030, 0.009 < 0.05, respectively) (**Figure 3**). The MOD of A. viscosus and P. intermedia cultured on BHI blood agar was higher than that cultured on BHI agar but was not significantly different (0.065 ± 0.033 vs. 0.044 ± 0.009; 0.113 ± 0.041 vs. 0.103 ± 0.033, respectively; P = 0.239, 0.685 > 0.05, respectively). The opposite was true for C. albicans, but without significant difference (0.042 ± 0.027 vs. 0.050 ± 0.016, P = 0.605 > 0.5). Comparison among groups showed that the MOD of P. intermedia cultured on BHI agar was higher than that of S. mutans (0.103 ± 0.033

Table 1. Relationship between fluorescence intensity (I) and bacterial concentration (C) with the 350 nm excitation wavelength

Species	Sheep blood	Maximum fluorescence intensity (I)					I-C linearly dependent coefficient, R
		C_0	$C_0/2$	$C_0/4$	$C_0/8$	$C_0/16$	
S. mutans	–	69.41	36.38	21.677	22.454	19.078	0.98
	+	177.567	72.849	49.926	32.877	23.345	0.89
S. sanguis	–	177.567	72.85	49.926	32.877	23.345	0.99
	+	648.528	399.264	128.93	56.389	24.903	0.99
A. viscosus	–	84.578	44.389	26.283	24.65	18.335	0.99
	+	159.397	67.324	61.387	51.582	54.664	0.94
P. intermedia	–	98.789	77.647	63.942	47.313	31.96	0.95
	+	79.48	34.78	30.178	30.867	47.702	0.79
L.acidophilus	–	281.746	128.595	80.719	41.962	30.403	0.99
	+	220.15	177.6	124.79	126.6	60.04	0.92
C.albicans	–	149.886	92.67	36.693	36.43	36.787	0.98
	+	99.13	48.17	35.71	23.527	28.899	0.98

Pearson correlation analysis was used to analyze the linear correlations between I and C using SPSS software; initial concentration, C_0, of each bacterium differs from each other. R > 0.8 indicates a strong linear correlation.

Table 2. Relative fluorescence energy at the 350 nm excitation wavelength

Species	Sheep blood	Sample	$Q_{432\text{-}440\ nm}/Q_{370\text{-}600\ nm} \times 100\%$ (x ± s)
S. mutans	–	5	0.062 ± 0.002
	+	5	0.068 ± 0.001**
S. sanguis	–	5	0.067 ± 0.004$^{\#}$
	+	5	0.079 ± 0.004**
A. viscosus	–	5	0.071 ± 0.005$^{\#\#}$
	+	5	0.069 ± 0.002
P. intermedia	–	5	0.070 ± 0.002$^{\#\#}$
	+	5	0.073 ± 0.001*
L. acidophilus	–	5	0.072 ± 0.001$^{\#\#}$
	+	5	0.081 ± 0.003**
C. albicans	–	5	0.070 ± 0.002$^{\#\#}$
	+	5	0.067 ± 0.005

$Q_{432\text{-}440\ nm} = \sum_{\lambda\text{-}432}^{\lambda\text{-}440} F(\lambda)\,d\lambda$; $Q_{370\text{-}600\ nm} = \sum_{\lambda\text{-}370}^{\lambda\text{-}600} F(\lambda)\,d\lambda$; *P < 0.05, **P < 0.01 vs. BHI agar; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 vs. S. mutans.

vs. 0.051 ± 0.015; P = 0.008 < 0.01). However, the other bacteria did not exhibit this significant difference, which implies that the MOD is not an ideal value for classifying bacterial species.

Fluorescence imaging characteristics

Fluorescence images of S. mutans, S. sanguis, A. viscosus, P. intermedia, L. acidophilus, and C. albicans were captured using a Nikon Eclipse 80i fluorescence microscope.

Candida albicans was the largest of the six microorganism, measuring 3-6 μm in diameter (**Figure 4**); the cells were round and grew in an aggregate pattern. Lactobacillus acidophilus was rod-shaped, 1 μm long, and with diffuse distribution, while S. sanguis was round, 1 μm in diameter, and had chain-like distribution. Actinomyces viscosus was rod-, V-, or Y-shaped with a diameter of 0.7 μm; P. intermedia was rod-shaped and 1-μm long; and S. mutans was round, with a diameter of 1 μm, and often had chain-like distribution.

Discussion

The excitation and emission wavelengths are necessary parameters of each bacterium when performing fluorescence spectrometry detection. Proper parameters can maximize detection sensitivity [20]. Following scanning of the absorption spectrum, we eventually selected 350 nm as the optimal excitation wavelength for the six studied bacteria because of its stability, which provided the foundation for subsequent detection.

Prevotella intermedia is a gram-negative bacterium, while S. mutans, S. sanguis, A. viscosus, L. acidophilus, and C. albicans are gram-positive. Most bacteria are autofluorescent: when illuminated with blue light at 407 nm, red auto-

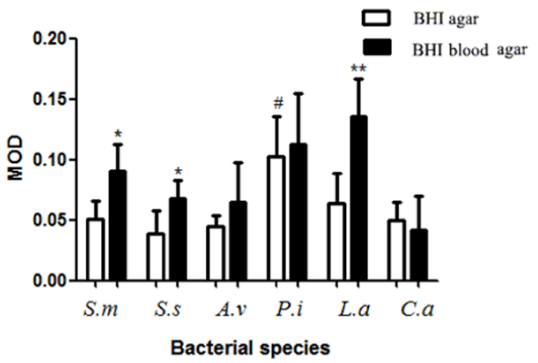

Figure 3. MOD of cariogenic bacteria. Mean values ± SEM, n = 5 for each bacterium, paired Student's t-test, *P < 0.05, **P < 0.01 vs. BHI agar; #P < 0.05 vs. S. mutans. S.m: S. mutans; S.s: S. sanguis; A.v: A. viscosus; P.i: P. intermedia; L.a: L. acidophilus; C.a: C. albicans

fluorescence from bacterial metabolites such as protoporphyrin IX are visible at 625-630 nm or 700 nm [21]. This spectral region is in the visible spectrum and has certain value in clinical applications [22]. Moreover, porphyrin compounds are present in most gram-negative bacteria but less so in gram-positive bacteria [23]. In contrast, we found that the gram-negative P. intermedia did not produce the red autofluorescence of protoporphyrin. However, all six bacteria produced blue fluorescence at 436 ± 4 nm with the 350 nm excitation wavelength. We believe that 436 ± 4 nm is a characteristic fluorescence peak position of these bacteria and the fluorescence does not originate from the porphyrin compound, which is more helpful when detecting gram-positive bacteria. In addition, sheep blood contains many exogenous fluorescence groups such as riboflavin and proteins [24]. However, we found little difference between the peak position value of bacteria cultured on BHI blood agar as compared to BHI agar, which proves that exogenous fluorescence groups may not influence fluorescence detection.

Although many fluorescence monomers contribute to bacterial autofluorescence, the fact that fluorescence intensity reflects the concentration of pure substances well [25] remained our basis for exploring the relationship between fluorescence intensity and bacterial concentration. The results in (**Table 1**) reveal that there were strong linear correlations between maximum fluorescence intensity and bacterial concentration between all studied bacteria except P. intermedia (R = 0.786). The linearly dependent coefficient, R, of P. intermedia was close to 0.8 (R = 0.786), and a linear trend between fluorescence intensity and bacterial concentration persisted despite the limited number of samples and accidental errors. Therefore, we conclude that it is feasible to estimate bacterial concentration by detecting the fluorescence intensity. The linear coefficient between fluorescence intensity and bacterial concentration is determined by fluorescence detection instruments and differs greatly between bacteria, so it is advisable to calculate the standard curves for each sample before further study.

As a relative value, $Q_{432\text{-}440\ nm}/Q_{370\text{-}600\ nm}$ (**Table 2**) is not related to bacterial concentration. That the $Q_{432\text{-}440\ nm}/Q_{370\text{-}600\ nm}$ of S. mutans, S. sanguis, P. intermedia, and L. acidophilus cultured on BHI blood agar was significantly higher than that of the control is consistent with the existing knowledge, as there are more fluorescence groups and more nutrients in blood agar. Abundant nutrients hasten bacterial growth and produce more metabolites, which contribute to stronger fluorescence. However, the $Q_{432\text{-}440\ nm}/Q_{370\text{-}600\ nm}$ of A. viscosus and C. albicans cultured on BHI blood agar and BHI agar did not differ significantly from each other, which may have been due to the small sample number and the bacterial properties. For example, as a fungus, C. albicans has strong growth ability and has fewer environmental requirements [26]. Comparison among groups showed that the $Q_{432\text{-}440\ nm}/Q_{370\text{-}600\ nm}$ of S. mutans cultured on BHI agar was significantly lower than that of the other bacteria. This indicates that the ability of S. mutans to produce fluorescence is weaker than the other bacteria, which may be a good means of differentiating between S. mutans and other bacteria.

The MOD is also irrelevant to bacteria concentration. The MOD of S. mutans, S. sanguis, P. intermedia, and L. acidophilus cultured on BHI blood agar was higher than that cultured on BHI agar (**Figure 3**). This result is consistent with that of the $Q_{432\text{-}440\ nm}/Q_{370\text{-}600\ nm}$ in (**Table 2**). Therefore, from our findings, both the MOD and $Q_{432\text{-}440\ nm}/Q_{370\text{-}600\ nm}$ reflect the fact that exogenous fluorescence groups in the culture environment may improve the fluorescence-producing ability in bacteria. From this experiment,

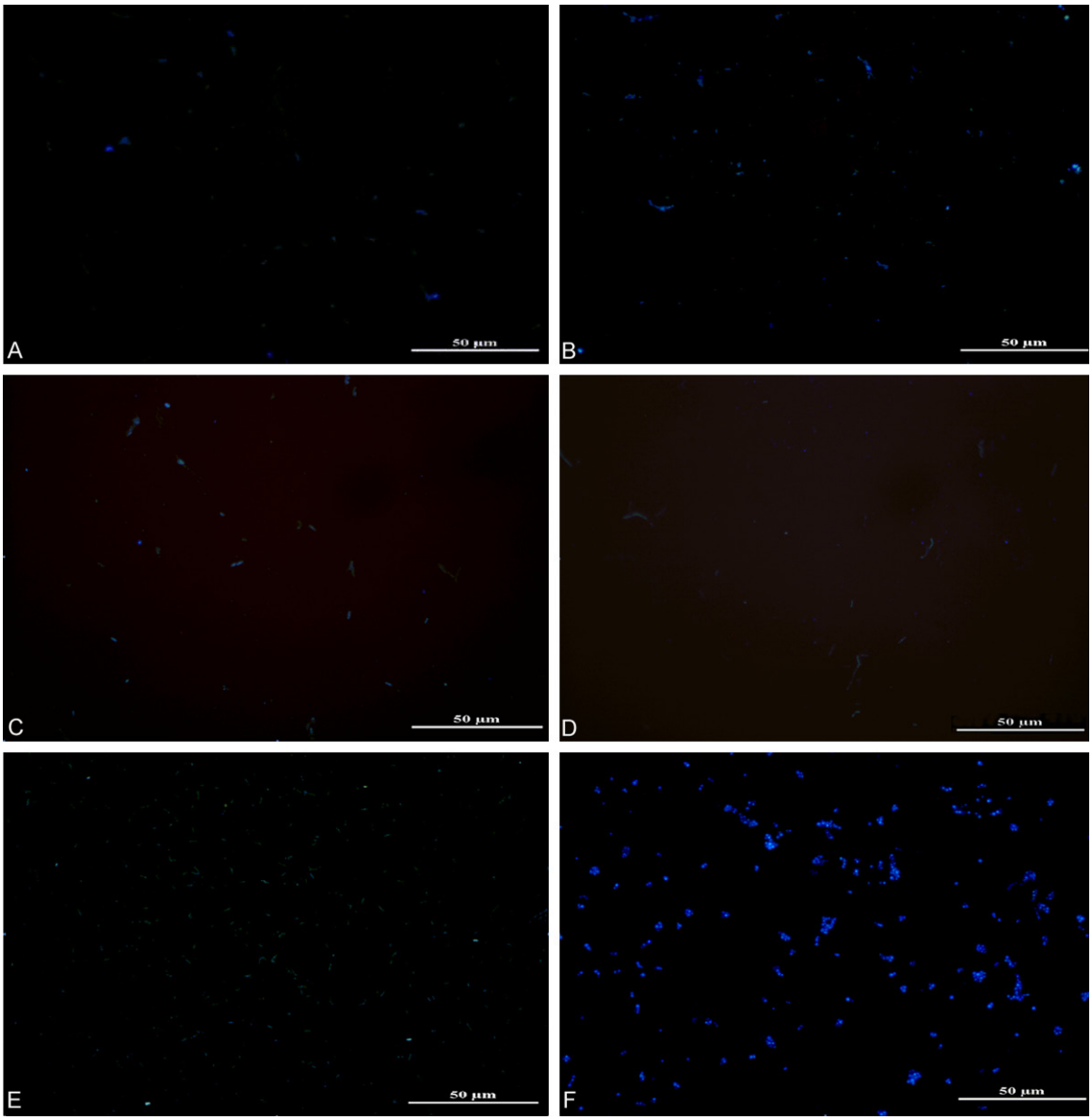

Figure 4. Fluorescence images of cariogenic bacteria (40×). (A: S. mutans; B: S. sanguis; C: A. viscosus; D: P. intermedia; E: L. acidophilus; F: C. albicans).

increased metabolism or endocytosis of the fluorescence groups generates greater fluorescence; however, we could not determine the main cause. To exclude nutrient interference, pure porphyrin compound can be used in place of sheep blood in follow-up experiments.

Unlike $Q_{432\text{-}440\ nm}/Q_{370\text{-}600\ nm}$, there was no significant difference between the MOD of the studied bacteria, which implies that it may not the ideal value for classifying bacterial species. However, when capturing the fluorescence microscopy images, we could not ensure the detection of all samples under the same circumstances. Long exposure time can easily cause fluorescence quenching and result in decreased fluorescence intensity. This is also a detecting defect in our experiment [27]. Sohn et al. [28] used principal component analysis (PCA) to analyze the fluorescence spectra of three different bacteria and performed a satisfactory classification. However, our experiment was limited by the number of samples and lack of repetition, therefore PCA was not used in the analysis. This can be improved to obtain more convincing results.

Conclusions

Under the optimal excitation wavelength of 350 nm, 436 ± 4 nm can be used as an indicator for detecting six common cariogenic bacteria. The fluorescence energy, Q, is a valuable index that reflects bacterial concentrations under fluorescence spectrometry detection. Exogenous fluorescence groups have greater influence on fluorescence intensity but little influence on fluorescence peak position detection.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hui Chen, Department of Conservative Dentistry and Periodontics, Affiliated Hospital of Stomatology, School of Medicine, Zhejiang University, 395 Yan An Road, Hangzhou 310006, Zhejiang, China. Tel: 0086-571-87-217437; Fax: 0086-571-87217437; E-mail: huic66@hotmail.com

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