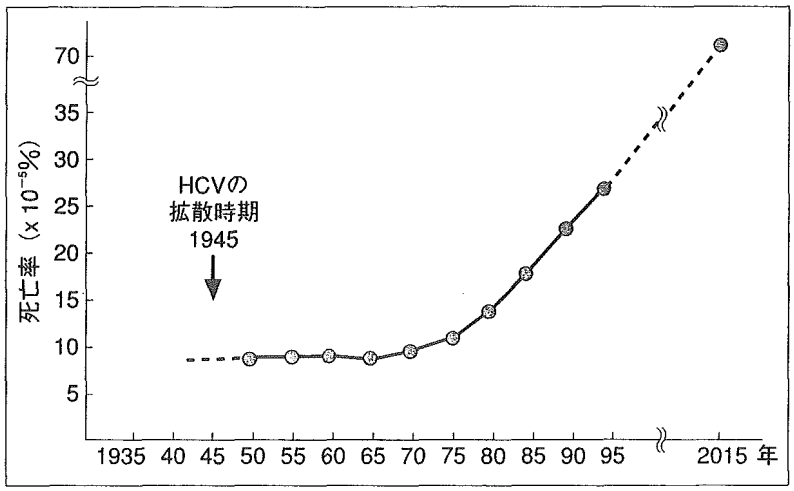


図❷ 日本のHCVの拡散時期からみたC型肝炎の1動向（Mizokami M. et al.：AASLD, 1999. より引用改変）

HCVの重複感染

HCVは、他のウイルスとも重複感染し、A型肝炎ウイルスやE型肝炎ウイルスのように急性肝炎を引き起こす場合は、重複感染により劇症化しやすいという報告がある[3]。HCVとHBVは、ともに血液を介して感染するため、重複感染例が少なくない。

HBs抗原陽性例（HBV感染者）の10%、HCV抗体陽性例（HCV感染者）の数%は重複感染例と考えられているが、実際にはもっと高頻度に起こっていると考えられている。しかし、HBVとHCVの同時感染による、慢性肝炎への影響はないと考えられている。HCVとHIVは同じ血液を介して感染するため、重複感染を起こす可能性も高い。

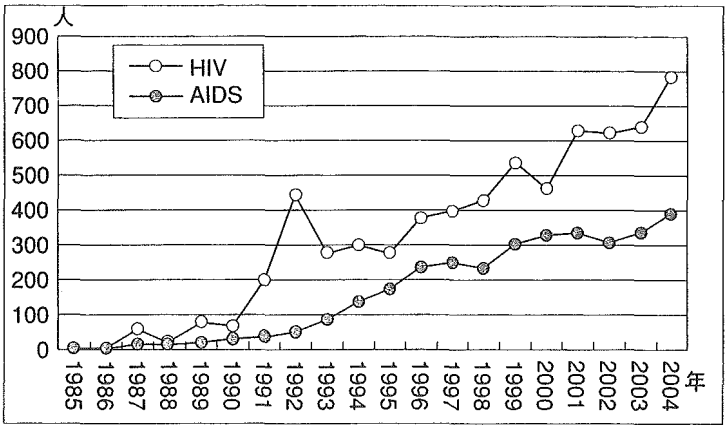
アメリカでは、HIV感染例の約30%（約30万人）がHCVに重複感染していると推定されている。日本では、まだ正確な数値が出ていないが、HIV感染例の約1/3が血液製剤によってHIVに感染していて、そのうちの97%がHIV・HCVの重複感染例である。またHIVの治療方法として多剤併用療法（HAART）の登場以降、HIV感染例の死因は従来に比べて大きく変化してきている。日和見感染症による死亡が減少し、肝疾患、とくにC型慢性肝炎とその合併症による死亡が増加してきている。

HIV

エイズの発生動向調査は1984年から開始され、日本におけるHIV感染者（AIDS未発症者）数とAIDS患者数がエイズ動向委員会により公表されている[4]。

1985年から2004年12月31日までの累積報告数（凝固因子製剤による感染例を除く）は、HIV感染者6,560、AIDS患者3,277となった。この他に血液凝固因子製剤によるHIV感染者1,434（生存中のAIDS患者167および死亡者564を含む）が報告されている（2003年5月31日現在）。

2004年に新たに報告されたHIV感染者は780（男698、女82）、AIDS患者は385（男344、女41）で、ともに2003年を大きく上回り過去最高となった（図3）。国籍・性別では日本国籍男性がHIV感染者全体の82%、AIDS患者全体の75%を占めている。ここ数年、日本国籍男性のHIV感染者が増加し続けている。2004年は日本国籍男性の同性間性的接触による感染がHIV感染者449、AIDS患者126ともに過去最高となった。とくに25～34歳群での報告数が顕著に増加している。日本国籍女性ではほとんどが異性間性的接触による感染であ



図❸　HIV感染者およびAIDS患者の年次推移

り、25～34歳群が多い。

累積調査において、静注薬物濫用や母子感染によるものはHIV感染者、AIDS患者いずれも1％以下であり、諸外国と比べわが国は少ない。HIV感染者は、すべての地域で増加しており、都道府県別では報告数が多い順に東京、大阪、神奈川、愛知、千葉、京都、静岡、兵庫、埼玉、長野、広島、沖縄、茨城である。

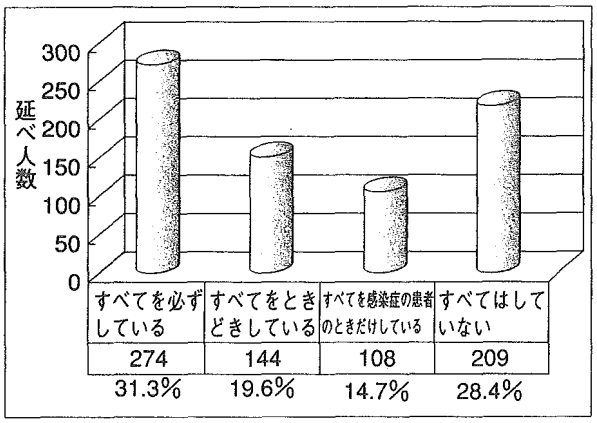
1999年3月31日までのAIDSによる死亡例は596で、日本国籍が485（男445、女40）、外国国籍が111（男77、女34）であった。献血者のHIV抗体陽性率は年々増加し続けている。2004年は、献血10万件あたり1.681（男2.629、女0.188）に達した。これらのデータをまとめると、HIV感染者数、献血者のHIV抗体陽性率はともに7年間で倍増している。よって、増加傾向に歯止めがかかっていない。

世界におけるHIV感染者は、2001年時点で4,000万人、年間感染者発生数は500万人と推定されている。これらの数値は、世界人口（約60億人）の約150人に1人が感染していることになる。日本の感染者は、約1億2,700万人のうちの1万人で約12,700人に1人が感染していることになり、世界の平均よりも1/84と少ない。地域別にみると、サハラ以南のアフリカで2,850万人（年間240万人）、南・東南アジアで500万人（年間50万人）、北アメリカで95万人（年間4.5万人）であり、年間感染者数をみても日本の感染者はまだまだ少ない。しかし、日本の感染者の増加傾向と世界の傾向をみると、今後急激に増えていくことが予測される。

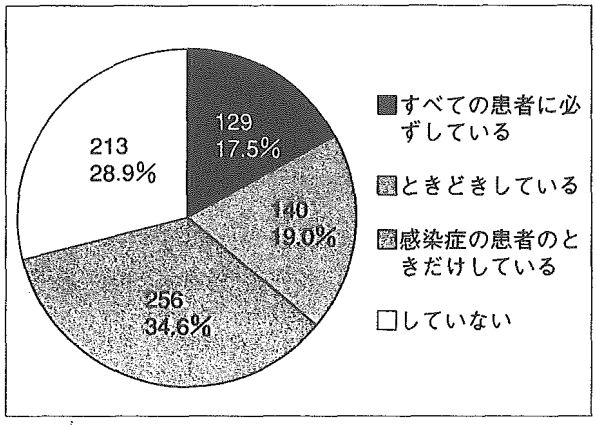
厚生労働省研究班の歯科医療における院内感染対策の研究活動

平成16年度厚生労働省科学研究において歯科医療における院内感染対策の研究班がスタートした。歯科医療を行うにあたって、その安全性の確保はもっとも重要な課題であるが、院内感染のリスクはいまだ減少しておらず、その監視体制の整備が望まれている。院内の環境や医用材料・医療機器に形成される細菌バイオフィルムは消毒薬に抵抗性を示し、そのことが院内感染の要因になっている可能性が高い。また、歯科治療による日和見菌やウイルスを含んだ唾液や血液の、直接的あるいは間接的な接触および飛沫による鼻粘膜および口腔粘膜への接触により感染症が起こる可能性もある。

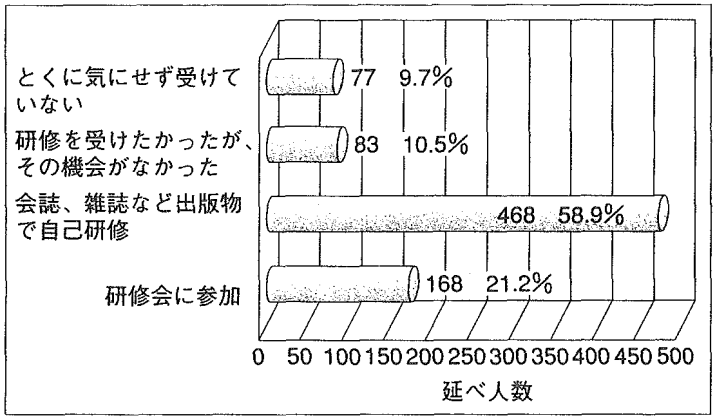
そこで、院内感染対策に対する意識や現状を把握するために一般歯科診療室を開業している歯科医師を対象にアンケート調査を実施し、院内感染対策を積極的に歯科医院に導入させるためになにが必要であるかを明らかにすることを目的とし、



図❹ 防護用メガネ（フェースシールド含む）、マスク、グローブのすべてを着用して診療していますか？



図❺ 患者ごとにハンドピースを交換していますか？



図❻ 歯科医療従事者の感染予防対策の研修状況についてお聞きします

研究を行った。今回はその一部のデータを用い研究成果を紹介する。

1．研究および方法

関東A県歯科医師会所属3,912人およびB県歯科医師会所属135人を対象にアンケート調査を行い、有効回答のあった742人（19%）および61名（45%）のアンケート結果を調査対象とした。院内感染に対する意識および知識に関する質問を行い、得られた回答から割合を算出した。また、研修を受けたグループと受けないグループに分け、それぞれの質問項目におけるグループ間の差を算出し、研修の効果について検討を行った。

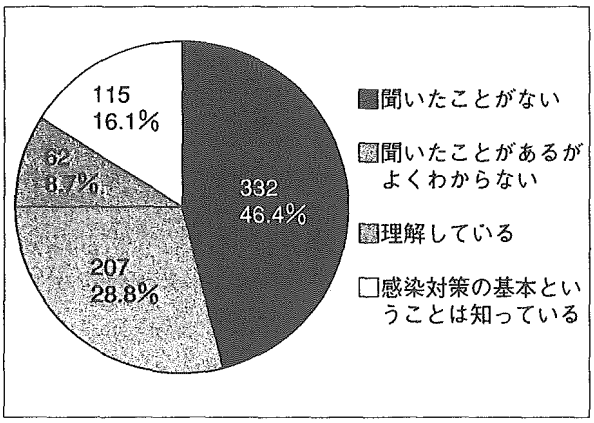
2．結果

“防護用メガネ（フェースシールド含む）、マスク、グローブのすべてを着用して診療しているか”との質問に、すべてを必ずしている31.3%、すべてをときどきしている19.6%、すべてを感染症の患者のときだけしている14.7%、すべてはしていない28.4%であった（図4）。

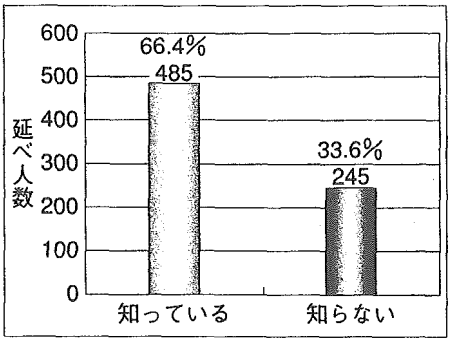
“患者ごとのハンドピースの交換”では、すべての患者に必ずしている17.5%、ときどきしている19.0%、感染症の患者のときだけしている34.6%、していない28.9%であった（図5）。

“感染予防対策の研修状況”では、研修会に参加21.2%、会誌、雑誌など出版物で自己研修58.9%、研修を受けたかったが、その機会がなかった10.5%、とくに気にせず受けていない9.7%であった（図6）。

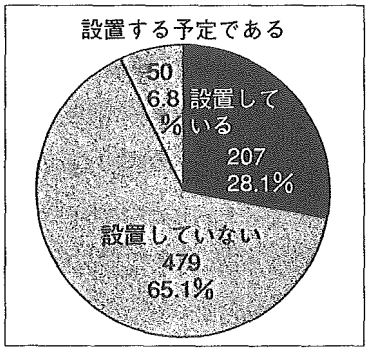
“ユニバーサルプリコーションとは何か”との質問には、聞いたことがない46.4%、聞いたことが



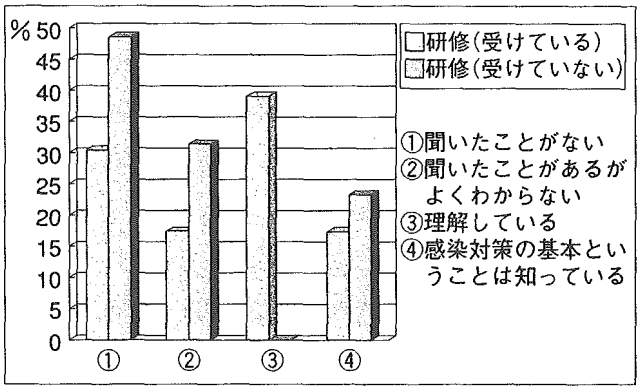
図❼ 「ユニバーサルプリコーション」とは何か知っていますか？



図❽ デンタルユニットのスリーウエイシリンジから出てくる水が水道水よりも細菌が多く含まれていることを知っていますか？



図❾ 自分の歯科医院内に口外バキュームを設置していますか？



図❿ 「ユニバーサルプリコーション」とは何か知っていますか？

あるがよくわからない28.8%、理解している8.7%、感染対策の基本ということは知っている16.1%と、回答者の75%以上が十分に理解しているとはいえない結果であった（図7）。

“デンタルユニットのスリーウェイシリンジから出てくる水が水道水よりも細菌が多く含まれていることを知っていますか”という質問に対しては、66.4%が知っていると答えた（図8）。

“自分の歯科医院内に口外バキュームを設置していますか”という質問に対しては、28.1%が設置していると答えた（図9）。

研修を受けたことがあるグループと受けたことがないグループに分け、院内感染対策の基本である“ユニバーサルプリコーションとは何か”という質問に対して理解していると答えたのは、研修を受けているグループで39.1%、研修を受けていないグループで0%であった（図10）。

3．考察

近年のHIV感染者の増加およびSARS、鳥インフルエンザなど新興感染症の流行、従来のB型、C型肝炎ウイルス感染等は、唾液や血液が飛び散りやすい歯科医療においてはなおのこと、このような微生物による感染者の来院に対応できる院内感染対策が必要である。しかし、一般歯科医院の院内感染対策の現状は、不十分と言わざるを得ない。とくに、院内感染対策の基本であるユニバーサルプリコーションを理解している割合が約10%と低く、情報量や知識不足に加えて、身近なこと、たとえばメガネやマスク、グローブのすべてを着用している歯科医が31.3%と低いことなどが問題点

として挙げられた。

ユニバーサルプリコーションを理解している割合が、研修を受けたグループで39.1%に対し、受けてないグループで0%と大きな差が認められたのは、驚くべき結果であった。これらの結果を踏まえ、情報発信および卒後研修システムの構築が重要な課題であると考えられた。

感染症は一度パンデミックになるスイッチが押されると瞬く間に流行地域が拡大し、世界各地で発症する危険性が出てくる。このような流行の原因として、交通や流通手段の進歩による人や動物の地球規模の移動や、地球環境の変化等、さまざま考えられている。

歯科においても、こうした近年の世界の感染症の発生動向を踏まえて医療に取り組んでいく必要があるが、現状の一般歯科医院の院内感染対策では不十分である。21世紀の感染症に対応できるような歯科医療を確立することをめざして、今後も研究を続ける必要があることを痛感する。

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COLUMN

手袋着用後の手洗い禁止

再使用のために手袋を洗浄してはいけない。これは未使用の手袋でも同じであるが、使用前に洗浄すべきではない。手袋は消毒薬により材質が劣化したり、洗浄動作でより穿孔が起こりやすくなるためである。再使用時の手洗いではさらにその確率は高くなる。

ディスポの手袋はあくまでディスポであり、再使用すべきではない。

処置前の洗口は何のため?

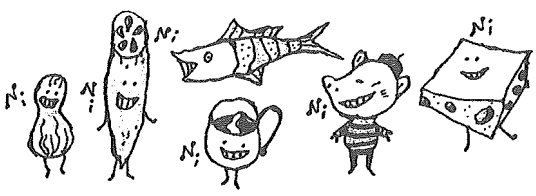
処置前の抗菌剤を用いての口腔洗浄は、細菌性心内膜炎の予防としてAHA(アメリカ心臓協会)から推奨されている。これにより、抜歯などの外科処置に起因する菌血症を減じることが目的である。また一方、これは処置に伴うエアロゾル中の病原微生物の量を少なくし、環境汚染や院内感染に対する予防策の意味合いもある。

きわめて理解しやすい理論ではあるが、しかし、これらはいずれも限定的な肯定の論文はあるものの、大規模研究による一般的なエビデンスは得られていないようである。ただ、処置前の洗口を否定する論文もなく、個人的にはおおいに行うべきと考える。

HIV感染者は黙って歯科にかかる?

依然、歯科におけるHIV感染者の診療拒否が続いていることから、厚労省から感染者の診療拒否の回避という異例の通達が出された。しかし、このHIV感染者の診療拒否は明らかにわかっている場合のことで、実は感染者と知らずに診療している例は枚挙にいとまがない。

そもそも感染者自身が自分の感染を認識していないことも多く、また黙ってかかっている場合もある。首都圏のエイズ拠点病院における2ヵ所でのアンケート結果では、いずれも80%以上が黙って歯科にかかった経験があると答えている。医療機関が感染を知れば拒否するし、感染者であっても知らなければ通常の患者同様ウエルカムとはまことに不思議なことである。



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Post-operative Infection by Pathogenic Micro-organisms in the Oral Cavity of Patients with Prostatic Carcinoma

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The aim of this study was to analyse the change in the oral cavity microflora of 14 patients who had undergone a radical prostatectomy for prostatic carcinoma. The detection of micro-organisms in the oral cavity was compared before and after the surgical procedure. Post-operative infection, defined as those patients who had increased Candida species counts and/or pathogenic bacteria only at the post-operative examination, was observed in 10 patients. Six patients showed increased Candida species counts at the post-operative examination compared with the pre-operative examination. In five patients, pathogenic bacterial species were detected at the post-operative examination but not at the pre-operative examination. One patient had detectable pathogenic bacterial species only at the post-operative examination along with increased Candida species counts. Our findings suggest that pre-operative oral hygiene to remove bacterial and Candida species from patients who are scheduled for surgical procedures is important for satisfactory clinical outcomes.

KEY WORDS: PROSTATIC CARCINOMA; OPPORTUNISTIC INFECTION; ORAL CAVITY; SURGERY; CANDIDA SPECIES

Introduction

In recent years, a new concept in micro-organism infection ·biofilm infection ·has been proposed.[1] Oral micro-organisms that attach to tooth or oral tissue surfaces aggregate in a hydrated extracellular polymeric substance (EPS) of their own synthesis to form biofilms, in which the micro-organisms colonize and coat the surface of the tooth or oral tissues.[2] Biofilms constitute a protected mode of growth that allows micro-organisms to survive in hostile environments, such as those containing antibiotics and when under attack from the immune system.[2] As a result,

biofilms can be the cause of many persistent and chronic infections.[2] Persistent oral infection has been thought to be the cause of infection and chronic inflammatory disease of various organs via periodontal tissue, oral membranes, the tonsils, the airway and the oesophagus.[3-5]
Oral biofilms in elderly people harbour opportunistic pathogens (such as *Enterobacter* species, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Candida* species, as well as commensal bacterial species including Gram-positive streptococci) causing dental caries and periodontal disease.[3,4] Several reports have suggested a relationship between decreased immunity and opportunistic infection of the oral cavity.[5-7]
Colonization of the oral cavity by pathogenic bacteria increases the risk of systemic disease, such as pneumonia and bacteraemia,[1,8] and infections can occur at non-operated sites in immunocompromised people after surgery.[9-12] Infection of the oral cavity could, therefore, be expected to occur after surgery at other sites within the body.
We performed this pilot study to investigate the pathogenic infection of the oral cavity in patients with prostatic carcinoma before and after they underwent a radical prostatectomy.

Patients and methods

PATIENTS

Subjects were patients with prostatic carcinoma who underwent radical prostatectomy at Okayama University Hospital (Okayama, Japan) between July 2002 and January 2004. Prior to the study, the study aims, design and procedures were explained, and informed consent was obtained from each patient. Ethical approval was not required for this study. As surgical prophylaxis, 1.5 g of ampicillin sulbactam or 1.0 g of cefazolin was given immediately before surgery, and the same antimicrobial agent at the same dosage was administered after surgery if the procedure had taken longer than 240 min. Ampicillin sulbactam or cefazolin, at the same dosage, was administered on the night of the surgical procedure and twice daily for 2 days post-operatively. Patients were starved overnight prior to the operation and they resumed normal eating and drinking from the day after the operation. None of the patients had undergone radiotherapy or chemotherapy.

BACTERIAL EXAMINATION

Supragingival plaque samples were collected from the postero-anterior buccal surface of the upper right second premolar and first molar using a cotton swab (Seedswab No. 1; Eiken, Tokyo, Japan) at 1 day before and 3 days after surgery. Sampling was performed by a doctor from the Urology Section (Okayama University Hospital) who had undergone training in the plaque sampling technique. Plaque samples were placed into transport fluid (0.4% agar and 0.15% thioglycollate/phosphate buffered saline) and transported to the Bio Medical Laboratory (Tokyo, Japan) for analysis to detect the following bacterial species: *Acinetobacter* species, *Citrobacter diversus*, *Citrobacter freundii*, *Enterobacter agglomerans*, *Enterobacter cloacae*, *Escherichia coli*, *Haemophilus parainfluenzae*, *Klebsiella oxytoca*, *K. pneumoniae*, methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staphylococcus aureus* (MSSA), *P. aeruginosa*, *Proteus mirabilis*, *S. marcescens*,

Streptococcus agalactiae and *Stenotrophomonas maltophilia*. Each plaque sample was placed directly onto chocolate, OPA Staphylococcus and Drigalski agar plates (Nippon Becton Dickinson, Kobe, Japan) using a stick. The plates were incubated in an atmosphere of 5% CO_2 in H_2 at 37 –C for 24 ·48 h. Representative microbial colonies from each plate were Gram stained and isolated by identification of their characteristic appearance, as well as by haemolytic, catalytic and oxidase reaction.[13] Isolates were suspended in 1 ml of 0.5% saline, gently shaken and tested in microbial detection kits (VITEK; BioM駸ieux Vitek Japan, Tokyo, Japan).[3,5] With regard to *Candida* species, quantitative analysis was performed by using fresh plaque samples from the swab, which were gently shaken in 3 ml of phosphate buffered saline for 5 min. The plaque samples were inoculated onto sabouraud dextrose agar plates using the EDDY JET spiral plating system (IUL, S.A. Torrent, Spain) and incubated for 48 h at 35–C to count *Candida* species colonies, which were identified by their characteristic morphological appearance and colour.

STATISTICAL ANALYSIS

The χ^2 test was used to assess statistical significance when comparing numbers for different categories. Differences at the $P < 0.05$ level were considered statistically significant. SPSS for Windows (Version 10.0, Chicago, IL, USA) was used for all statistical analyses.

Results

PATIENT CHARACTERISTICS

The clinical and surgical characteristics of the 14 patients enrolled in this study are shown in Table 1. The average age of the patients was 65.5 years (50 ·59 years, $n = 3$; 60 ·69 years, $n = 6$; 70 ·79 years, $n = 5$). Seven patients had complications (hypertension, $n = 5$; diabetes mellitus, $n = 3$; angina pectoris, $n = 1$; and pulmonary emphysema, $n = 1$). Radical prostatectomy was performed via an open ($n = 10$) or laparoscopic ($n = 4$) operation. The duration of surgery was between 175 and 380 min (mean, 284 min).

ORAL MICROFLORA PRE- AND POST-SURGERY

Micro-organisms detected in the oral cavities of patients in this study are shown in Table 2. The detection rate of *Candida* species at the pre- and post-operative examinations was 35.7% (five of 14) and 57.1% (eight of 14), respectively. After surgery, six patients (42.9%) showed a logarithmic increase in *Candida* species counts (measured as colony-forming units [CFU]). Before surgery, three patients (21.4%) possessed pathogenic bacterial species in oral cavities. Of 11 patients who did not have pathogenic bacterial species in their oral cavities at the pre-operative examination, five patients had pathogenic bacteria (*E. cloacae*, $n = 2$; *P. aeruginosa*, $n = 1$; *Acinetobacter* species, $n = 1$; *C. freundii*, $n = 1$; *K. pneumoniae*, $n = 1$ and coagulase-negative *Staphylococcus* species, $n = 1$) at the post-operative examination. Of these five patients, four did not show increased CFU counts of *Candida* species at the post-operative examination. In one patient (patient 8), *E. cloacae* and *K. pneumoniae* were detected only at the post-operative examination along with increased *Candida* species counts.

POST-OPERATIVE INFECTION AND THE RELATIONSHIP WITH AGE, COMPLICATIONS AND DURATION OF SURGERY

As shown in Table 3, the distribution of patients who demonstrated increased *Candida* species counts, or who had detectable pathogenic bacterial species only at the post-operative examination, was investigated with regard to relationship to age, presence of complication(s) and duration of surgery. No significant differences were observed between the two groups within each category.

Discussion

Candida species and pathogenic bacterial species were detected more frequently at the post-operative examination than at the pre-operative examination. These micro-organisms have been reported to cause opportunistic infections.[14-18] Decreased immunity may result in infection by these micro-organisms, and surgical procedures are thought to increase the risk of infection by decreasing immunity.[9,10] Radical prostatectomy may decrease the immune function of patients, resulting in a change in their oral microflora; in addition, long-term administration of antibiotics may also cause opportunistic infections. In this study, each post-operative examination was performed 3 days after the operation in order to minimize the effects of prophylactic antibiotics. Infection was not considered to be a direct result of the surgery because of the distance between the operation site and the oral cavity.

Cross-sectional studies have reported that opportunistic infections of the oral cavity occur in people who seem to have a decreased immune function. *Candida* species levels were higher in the oral cavities of critically ill patients than in women who were considered to be healthy.[19] Smith *et al.*[20] reported that coagulase-negative *Staphylococcus* species emerged in many debilitated elderly patients and in those with oral Crohn 痴 disease. The nutritional status has also been reported to be related to the detection of MRSA.[7] Senpuku *et al.*[5] found that several pathogenic micro-organisms were isolated at a significantly higher rate in functionally dependent elderly people with heart disease than in those that were functionally independent. A longitudinal study investigating the relationship between oral micro-organisms and general health has not been performed. This is the first report to examine the change in oral cavity microflora before and after surgery involving organs that are not in the oropharyngeal region. Our study provides novel information about the influence of surgery on oral microflora.

Increased *Candida* species counts were not observed at the post-operative examination in the majority of patients who demonstrated detectable pathogenic bacterial species at the post-operative examination. One patient had detectable levels of *E. cloacae* and *K. pneumoniae* at the post-operative examination along with increased *Candida* species counts, which suggested that the optimal condition for *Candida* species growth was probably different to that preferred by pathogenic bacterial species. The growth of oral microflora is likely to be dependent on the condition of the oral cavity.

Micro-organisms detected at the post-operative examination in this study have been reported to cause bacteraemia and several diseases in other organs via transmission through the bloodstream.[21,22] In the case of patients with periodontal disease, these micro-organisms in the oral cavity

can invade in the bloodstream by gingival bleeding, and a relationship between septicaemia and periodontitis has been suggested.[23-25] Micro-organisms such as *Candida* species, *P. aeruginosa*, *Acinetobacter* species, *K. pneumoniae*, and *C. freundii* in the oral cavity might cause pneumonia by aspiration.[26] Furthermore, micro-organisms detected in this study have been reported to cause nosocomial infection.[27,28] An antiseptic decontamination of the dental plaque with a 0.2% chlorhexidine gel decreased dental bacterial colonization and reduced the incidence of nosocomial infection in intensive care unit patients exposed to mechanical ventilation.[29] Considerable attention needs to be paid to oral biofilms and the oral hygiene of patients prior to surgery, even when the surgical site is some distance from the oral cavity. Our study suggests that good oral care of patients who are scheduled for surgery is important for satisfactory post-operative management.

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Conflicts of interest
No conflicts of interest were declared in relation to this article.

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Table 1 Characterization of subjects

No	Age	Complication	Operation methods	Operation time (min)
1	63	No	Open	265
2	74	No	Laparoscopy	270
3	64	No	Open	250
4	68	Hypertension Angina pectoris	Open	300
5	67	No	Laparoscopy	355
6	70	Diabetic mellitus	Open	255
7	59	Hypertension Diabetic mellitus	Laparoscopy	380
8	74	Hypertension Pulmonary emphysema	Open	175
9	50	No	Laparoscopy	380
10	72	No	Open	375
11	73	Hypertension	Open	255
12	66	Hypertension	Open	245
13	63	Diabetic mellitus	Open	245
14	54	No	Open	220

Table 3 Distribution of subjects with postoperative infection by age, complications and operation time

Categories	Number of subjects (%)	P-value
Age		
≤69	6 (66.7)	0.597
≥70	4 (80.0)	
Complications		
No	4 (57.1)	0.232
Yes	6 (85.7)	
Operation time (min)		
<300	6 (66.7)	0.597
≥300	4 (80.0)	

Table 2 List of detected bacteria and the levels of detection for *Candida* spp. at preoperative and postoperative examination

No	Pathogenic bacteria		Colony-forming unit of *Candida* spp.	
	Preoperative	Postoperative	Preoperative	Postoperative
1	ND	ND	ND	305
2	ND	ND	ND	13902
3	ND	ND	ND	ND
4	ND	*Citrobacter freundii* *Acinetobacter spp*	26258	2134
5	ND	ND	ND	ND
6	ND	*Enterobacter cloacae*	ND	ND
7	ND	*Pseudomonas aeruginosa*	8780	ND
8	ND	*Enterobacter cloacae* *Klebsiella pneumoniae*	671	70122
9	*Serrathia marcescens* *Pseudomonas aeruginosa*	*Serrathia marcescens*	ND	3110
10	ND	ND	488	34091
11	ND	ND	ND	ND
12	ND	Coagulase negative- *Stapylococcus* spp.	ND	ND
13	*Klebsiella pneumoniae* *Enterobacter cloacae*	*Klebsiella pneumoniae*	ND	143293
14	*Enterobacter cloacae*	*Enterobacter cloacae*	61	61

ND: not detected

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Original Article

Clinical Implications of Biofilm Formation by *Enterococcus faecalis* in the Urinary Tract

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The potential relationships between biofilm formation and pathogenicity of *Enterococcus faecalis* in urinary tract infections (UTI) were investigated. Over a 12-year period from 1991 through 2002, a total of 352 *E. faecalis* isolates were collected from patients with complicated UTI (one isolate per patient) at the urology ward of Okayama University Hospital. We analyzed the prevalence and transferability of genes encoding virulence factors (*asa1*, *esp*, *cylA*, *gelE/sprE*) and antimicrobial resistance (*aac(6')/aph(2'')*). The production of biofilm, hemolysin and gelatinase by these isolates was also examined and the associated medical records of patients were retrospectively reviewed. Of 352 *E. faecalis* isolates, 315 possessed *asa1* and/or *esp* genes. Of the 63 hemolysin- and 167 gelatinase-producing isolates, 59 and 94 isolates, respectively, possessed both *asa1* and *esp* genes. *E. faecalis* isolates with both *asa1* and *esp* genes formed biofilms at significantly higher rates than those with neither gene ($P = 0.038$). The genes encoding *asa1*, *cylA* and *aac(6')/aph(2'')* were transferable and appeared to have accumulated in these isolates. The *E. faecalis* isolates possessing *asa1* and/or *esp* genes were found from both catheter-related or -unrelated UTI. Our study indicates that *E. faecalis* isolates that have accumulated virulence genes are apt to form persistent biofilms in the urinary tract.

Key words: *Enterococcus faecalis*, urinary tract infection, biofilm formation, pathogenicity, gene transfer

Enterococcus faecalis is a normal commensal in the human intestinal flora but can lead to nosocomial infections [1-5]. Although the pathogenicity of *E. faecalis* in the urinary tract is not considered high, *E. faecalis* has been isolated from the urinary tract at increasing frequencies: up to 20% of urinary isolates in some reports [6-8]. Several virulence factors have been described in *E. faecalis*, including aggregation substance (Agg), enterococcal surface protein (Esp), cytolysin (Cyl) having both hemolytic and bactericidal activity, and gelatinase (Gel) [1, 3, 4]. These factors have been thought to act synergistically to enhance virulence by facilitating achievement of a quorum and activating the quorum-sensing mode of regulation, resulting in tissue damage and potentially deeper tissue invasion [1, 9-12]. Recent studies have shown that enterococci form biofilms and that the *E. faecalis fsr* quorum-sensing system controls biofilm development [13-17].

Agg is a surface protein expressed by the *asa1* gene, which is located on pheromone-responsive *E. faecalis* plasmids [18, 19]. It is produced in response to pheromones secreted by potential recipient *E. faecalis* cells,

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and causes the aggregation of donor and recipient cells, thereby facilitating the transfer of plasmids that may carry virulence traits and antibiotic resistance genes [18, 19]. Agg may also play an important role in the onset of enterococcal infection by facilitating the adherence of enterococci to cardiac vegetations as well as to the epithelial cells of the intestine, kidney and urinary tract [1]. Esp encoded by the chromosomal *esp* is associated with increased virulence, colonization and persistence in the urinary tract, along with biofilm formation [1, 13, 17, 20, 21]. The production of Cyl has also been shown to significantly worsen the severity of endocarditis and endophthalmitis in animal models as well as to contribute to the severity of enterococcal disease in humans [1, 22]. Cyl is either encoded within pheromone-responsive plasmids or on the chromosome within pathogenicity islands and is transcribed as an operon that contains at least 5 genes including *cylA* [1, 22]. Gel, encoded by the chromosomal *gelE*, is an extracellular zinc-metalloprotease that hydrolyzes collagen, gelatin, and small peptides and that has been shown to exacerbate endocarditis in an animal model [1].

In the present study, we investigated the potential relationships between biofilm formation and clinical implications of *E. faecalis* isolates in the urinary tract. Over a 12-year period from 1991 through 2002, a total of 352 *E. faecalis* isolates were collected from patients with complicated urinary tract infection (UTI) at the urology ward of Okayama University Hospital. We analyzed the prevalence and transferability of genes encoding virulence factors (*asa1*, *esp*, *cylA*, *gelE/sprE*) and antimicrobial resistance (*aac(6')/aph(2")*). In addition, the production by these isolates of biofilm and extracellular enzymes, hemolysin (Hln) and Gel was examined, and the associated medical records of the patients were retrospectively reviewed. The data were summarized in 4 groups based on the presence/absence of *asa1* and *esp* genes encoding enterococcal adhesins, Agg and Esp, respectively.

Materials and Methods

Bacterial isolates from patients with UTI. The *E. faecalis* bacterial isolates used in this study were isolated from patients with complicated UTI at the Department of Urology, Okayama University Hospital, over a 12-year period from 1991 through 2002. A total of 352 isolates that grew to $\geq 10^4$ CFU/ml in urinary culture were selected for this study. All 352 patients (one isolate per patient) had documented pyuria (WBC ≥ 5/hpf).

Polymerase chain reaction (PCR) assay. The presence of *asa1*, *esp*, *cylA* *gelE/sprE* and *aac(6')/aph(2")*, which encode Agg, Esp, Cyl, Gel/serine protease and gentamicin resistance, respectively, was confirmed by PCR assay. The primers and PCR conditions used in this study are summarized in Table 1. Multiplex PCR assay was used to detect the *asa1* and *cylA* genes using primers reported by Huycke *et al.* [23]. Primers reported by Shankar *et al.* [20] were used for amplification within the N-terminal region of *esp*. Primers reported by Nakayama *et al.* [24] and Van de Klundert *et al.* [25] were used to amplify *gelE/sprE* and *aac(6')/aph(2")* genes, respectively. DNA amplification was carried out by the method of Kariyama

Table 1 PCR primers and conditions used in this study

Primer specificity	Primer sequences	Product length [bp]	PCR conditions				Reference
			Initial denaturation	Cycling	Cycle	Final extention	
asa1	F: 5'-GATTCTTCGATTGTGTTGTAAACG-3' R: 5'-GGTGCCACAATCAAATTAGG-3'	380	2 min, 95 °C	1 min, 95 °C; 1 min, 46 °C; 1 min, 72 °C	35	10 min, 72 °C	23
esp	F: 5'-TTGCTAATGCTAGTCCACGACC-3' R: 5'-GCGTCAACACTTGCATTGCCGAA-3'	955	2 min, 95 °C	45 sec, 94 °C; 45 sec, 63 °C; 2 min, 72 °C	30	7 min, 72 °C	20
cylA	F: 5'-GGGGATTGATAGGCTTCATCC-3' R: 5'-GCACCGACGGTAATTACAGACTCTAG TCCTCC-3'	432	2 min, 95 °C	1 min, 95 °C; 1 min, 46 °C; 1 min, 72 °C	35	10 min, 72 °C	23
gelE/sprE	F: 5'-ATGAAGGGAAATAAAATTTTATAC-3' R: 5'-CTGCTGGCACAGCGGATA-3'	2428	2 min, 94 °C	30 sec, 94 °C; 30 sec, 48 °C; 3 min, 72 °C	35	6 min, 72 °C	24
aac(6')/aph(2")	F: 5'-CCAAGAGCAATAAGGGCATA-3' R: 5'-CACTATCATAACCACTACCG-3'	220	5 min, 94 °C	1 min, 94 °C; 1 min, 55 °C; 1 min, 72 °C	35	10 min, 72 °C	25

et al. [26]. Briefly, total cellular DNA was prepared as follows: 0.5 ml of *E. faecalis* culture, grown overnight in Todd Hewitt broth (Becton Dickinson and Company, Sparks, MD, USA), was centrifuged, and the pellet was resuspended in 50 μl of InstaGene (Bio-Rad Laboratories, Hercules, CA, USA). After the suspension was heated for 10 min at 100 °C, 2.5 μl of the supernatant was mixed with 22.5 μl of prepared reaction mixture to start the reaction. The primer pairs were added to the respective reaction mixtures. The 25-μl reaction volume contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 0.625 U of *Taq* DNA polymerase (Takara Shuzo, Shiga, Japan). PCR products were analyzed by electrophoresis on a 2% agarose gel. After electrophoresis, gels were stained with ethidium bromide (1 mg/l) and photographed under a UV trans-illuminator. A 100-bp DNA Ladder (New England Biolabs, Beverly, MA, USA) was used as a molecular size marker. The fragment sizes of PCR products are shown in Table 1.

Detection of hemolysin-producing isolate. Production of hemolysin was determined by plating *E. faecalis* isolates onto Todd Hewitt agar plates supplemented with 5% rabbit blood and incubated at 37 °C for 48 h. When hemolysis was observed on the plate at 48 h, the isolate was considered a hemolysin-producing isolate.

Detection of gelatinase-producing isolate. Production of gelatinase was determined by the method of Su *et al.* [27]. A transparent halo around colonies after exposure to a solution saturated with ammonium sulfate on the surface of the medium was considered a gelatinase-positive response.

Biofilm formation assay. *E. faecalis* isolates were grown overnight at 37 °C in tryptic soy broth supplemented with 0.25% glucose [15]. The culture was diluted 1:100 in medium, and 200 μl of this cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtiter plates (Corning Inc., Corning, NY, USA). After 24 h at 37 °C without shaking, wells were gently washed three times with 300 μl of distilled water, dried in an inverted position, and stained with 300 μl of 2% crystal violet solution in water for 45 min. After staining, plates were washed 3 times with distilled water. Quantitative analysis of biofilm production was performed by adding 300 μl of ethanol-acetic acid (95:5, vol/vol) to destain the wells. One hundred microliters from each well was transferred to a new microtiter plate, and the level (optical density; OD) of crystal violet present in the destaining solution was measured at 570 nm using a microtiter plate reader (Seikagaku Co., Tokyo, Japan). Each assay was performed in triplicate. As a control, uninoculated medium was used to determine background OD. The mean OD_{570} value from the control wells was subtracted from the mean OD_{570} value of tested wells.

Conjugative transfer experiments. Mating experiments were performed by the method of Clewell *et al.* [28]. Each of 43 *E. faecalis* isolates possessing the 3 genes *asa1*, *cylA* and *aac(6')/aph(2")* was used as donor, and *E. faecalis* FA2-2 (rifampicin and fusidic acid resistance) was used as recipient. Broth matings were performed with a donor/recipient ratio of 1:10. Overnight cultures of 0.05 ml of donor and 0.5 ml of recipient were added to 4.5 ml of fresh broth, and the mixtures were incubated at 37 °C with gentle agitation for 4 h. Portions (0.1 ml) of the mixed and diluted culture were then plated on Todd Hewitt agar plates with appropriate selective antibiotics. Transconjugants were selected on Todd Hewitt agar plates supplemented with 500 μg of gentamicin/ml and 25 μg of rifampicin and fusidic acid/ml. Colonies were counted after 48 h of incubation at 37 °C. Separate platings where donors alone were selected provided a basis for estimating the transfer frequency (per donor). Filter matings were also carried out. Overnight cultures of 0.1 ml of donor and 1 ml of recipient were added to 9 ml of fresh broth, and the mixtures were immediately collected on a membrane (25 mm width 0.45 μm pore size filter, type HA; Millipore Corp., Billerica, MA, USA), which was inverted onto the surface of a Todd Hewitt agar plate supplemented with 4% horse blood. After incubation at 37 °C for 20 h, the cells were suspended in 1 ml of Todd Hewitt broth. The subsequent procedure was the same as that for broth matings. Transfer frequencies were calculated as the number of transconjugants per donor cell.

Retrospective clinical study. We retrospectively reviewed the medical records of the 352 patients whose characteristics were summarized in Table 2 and classified their UTI as catheter-related or catheter-unrelated, polymicrobial or monomicrobial, and febrile or non-febrile cases. Febrile UTI was defined as UTI in a patient with a body temperature of $\geq$ 37.0 °C.

Statistical methods. Data are expressed as mean values $\pm$ standard deviation (SD). Comparison of OD_{570} values between groups was carried out using

Table 2 Demographic and clinical characteristics of 352 patients with UTI due to *E. faecalis*

Characteristics	Value
Age; median ± SD (range)	61 ± 20.7 (0-94)
Sex; no. male/no. female	240/112
Polymicrobial infections	257 isolates
Pseudomonas aeruginosa	42
Escherichia coli	28
Methicillin-resistant *Staphylococcus aureus*	22
Klebsiella pneumoniae	19
Serratia marcescens	17
Staphylococcus epidermidis	13
Citrobacter freundii	13
Staphylococcus aureus	9
Candida albicans	7
Proteus mirabilis	6
Others	81
Underlying diseases	
Bladder cancer	90
Prostatic cancer	49
Other urinary tract cancer	10
Neurogenic bladder	79
Benign prostatic hyperplasia	40
Urinary tract stones	19
Ureteral stricture	7
Ureteropelvic junction stenosis	6
Vesicoureteral reflux	5
Others	47

Fisher's exact test or Mann-Whitney's U test. All results were considered statistically significant at the $P < 0.05$ level.

Results

Presence of various genes, and the production of extracellular enzymes in E. faecalis isolates. Of the 352 *E. faecalis* isolates, 291 (82.7 %), 254 (72.2%), 164 (46.6%), 306 (86.9%), and 141 (40.1%) isolates possessed *asa1*, *esp*, *cylA*, *gelE/sprE*, and *aac(6')/aph(2")*, respectively. Of 164 isolates possessing the *cylA* gene, 63 (38.4%) isolates produced Hln. Of 306 isolates possessing *gelE/sprE* genes, 167 (54.6 %) isolates produced Gel. The number of *E. faecalis* isolates with both *asa1* and *esp* genes, with *asa1* gene only, with *esp* gene only, and with neither gene were 230, 61, 24 and 37, respectively.

Biofilm formation of E. faecalis isolates. Of the 352 *E. faecalis* isolates, 64 (18.2%), 156 (44.3 %), and 132 (37.5%) isolates exhibited strong ($OD_{570} \geq 0.5$), medium ($OD_{570} \geq 0.2$ to < 0.5), and weak (OD_{570} 0 to < 0.2) biofilm formation, respectively. The mean OD_{570} of the 352 isolates was 0.36 ± 0.37 (mean ± SD). We evaluated the relationships between biofilm formation and the 4 virulence determinants of the *E. faecalis* isolates. As shown in Table 3, the mean OD_{570} value (mean ± SD) was significantly higher in *asa1*-, *esp*-, and *cylA*-positive isolates than in *asa1*-, *esp*-, and *cylA*-negative isolates ($P = 0.0176$, $P = 0.0276$ and $P = 0.0116$, respectively). The value was also significantly higher in Hln producing isolates than in Hln non-producing isolates ($P = 0.0384$). We also evaluated the biofilm-forming capacities of *E. faecalis* isolates in the 4 groups based on the presence/absence of *asa1* and *esp* genes (Fig. 1). As shown with a box and whisker plot, the *E. faecalis* isolates with *asa1* and *esp* genes had greater capacities for biofilm formation than did those lacking these genes. The Mann-Whitney's U test of 2 mean OD_{570} values (mean ± SD), 0.41 ± 0.42 and 0.22 ± 0.16, in 230 *asa1*-, *esp*- positive and 37 *asa1*-, *esp*- negative isolates, respectively, confirmed that the *E. faecalis* isolates possessing both *asa1* and *esp* genes had significantly greater capacities for biofilm formation than did those lacking both genes ($P = 0.038$).

Percentage of E. faecalis isolates possessing cylA, gelE/sprE and aac(6')/aph(2") genes in 4 groups based on the presence/absence of asa1 and esp genes. As shown in Fig. 2, the percentage of *E. faecalis* isolates possessing *cylA* and/or *aac(6')/aph(2")* genes was greatest in the group with both *asa1* and *esp* genes and lower in the groups with only the *asa1* gene or the *esp* gene. No isolates possessing the *cylA* gene and only 1 isolate possessing the *aac(6')/aph(2")* gene were found in the group lacking both the *asa1* and *esp* genes. In contrast, *E. faecalis* isolates possessing *gelE/sprE* genes were found evenly among the 4 groups. Of the 230 *E. faecalis* isolates in the group with *asa1* and *esp* genes, 147 (63.9 %), 195 (84.8%) and 117 (50.9%) possessed *cylA*, *gelE/sprE* and *aac(6')/aph(2")* genes, respectively. Of the 61 *E. faecalis* isolates in the group with only the *asa1* gene, 15 (24.6%), 56 (91.8%) and 22 (36.1%) possessed the *cylA*, *gelE/sprE*, and *aac(6')/aph(2")*genes, respectively. Of the 24 *E. faecalis* isolates in the group with only the *esp* gene, 2 (8.3%), 23 (95.8%) and 1 (4.2 %) possessed the *cylA*, *gelE/sprE*, and *aac(6')/aph(2")* genes, respectively. Of the 37 *E. faecalis*

Table 3 Relationship between biofilm-forming capacities and virulence factors/clinical background

	Number of isolates	OD_{570} (mean ± SD)	*P* value (Mann-Whitney's *U* test)
Total isolates tested	352	0.36 ± 0.37	
Virulence determinants			
asa1-positive	291	0.38 ± 0.38	0.0176
asa1-negative	61	0.27 ± 0.27	
esp-positive	254	0.40 ± 0.41	0.0276
esp-negative	98	0.26 ± 0.18	
cylA-positive	164	0.41 ± 0.41	0.0116
cylA-negative	188	0.32 ± 0.32	
gelE/sprE-positive	306	0.36 ± 0.35	0.0915
gelE/sprE-negative	46	0.35 ± 0.46	
Extracellular enzymes			
hemolysin producing	63	0.47 ± 0.48	0.0384
hemolysin non-producing	289	0.34 ± 0.33	
gelatinase producing	167	0.35 ± 0.31	0.1376
gelatinase non-producing	185	0.37 ± 0.42	
Clinical background			
catheter-related	107	0.33 ± 0.34	0.0582
catheter-unrelated	245	0.38 ± 0.38	
polymicrobial	202	0.35 ± 0.34	0.5505
monomicrobial	150	0.37 ± 0.40	
febrile	60	0.31 ± 0.32	0.1267
non-febrile	292	0.37 ± 0.38	

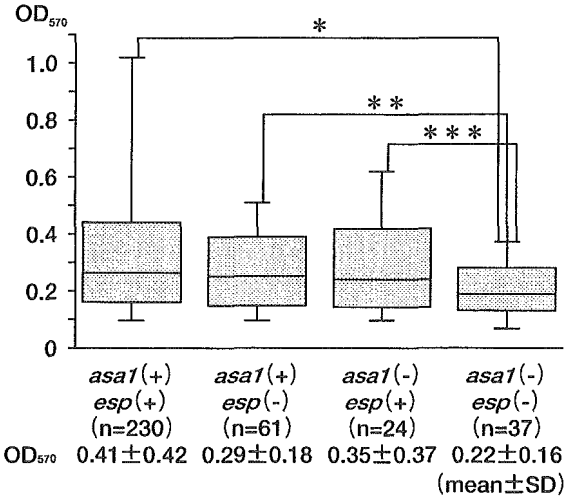

Fig. 1 Biofilm-forming capacities of *E. faecalis* isolates in 4 groups based on the presence/absence of *asa1* and *esp* genes. OD_{570} values of the isolates in the 4 groups are shown by the box and whiskers plot, which represents a five-number summary (upper extreme, upper quartile, median, lower quartile, and lower extreme). The mean OD_{570} values (mean ± SD) of the 4 groups are also shown.
*$P = 0.038$; **$P = 0.0449$; ***$P = 0.1208$ (Mann-Whitney's *U* test).

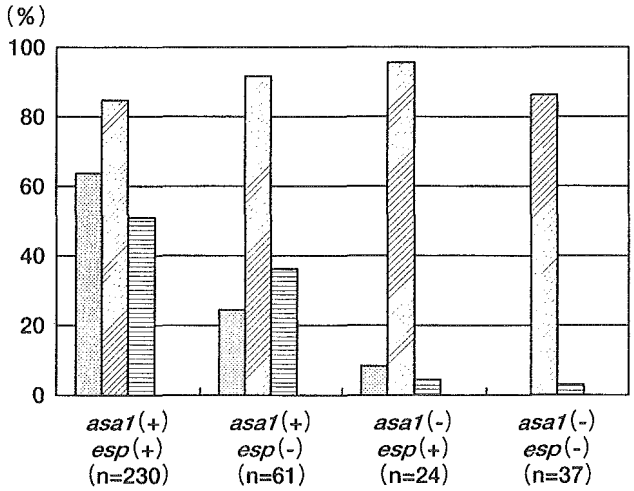

Fig. 2 Percentage of *cylA*-, *gelE/sprE*- and *aac(6')/aph(2'')*-positive isolates among *E. faecalis* isolates in 4 groups based on the presence/absence of *asa1* and *esp* genes.
Bars: ⁘, *cylA*; ▨, *gelE-sprE*; ▤, *aac(6')-aph(2'')*

isolates in the group with neither gene, 0 (0%), 32 (86.5 %) and 1 (2.7%) possessed the *cylA*, *gelE/sprE*, and *aac(6')/aph(2")* genes, respectively.

Number of E. faecalis isolates producing hemolysin and gelatinase in 4 groups based on the presence/absence of asa1 and esp genes. As shown in Fig. 3, the majority of Hln-producing and Gel-producing isolates were found in the group with both *asa1* and *esp* genes. Of the 63 Hln-producing and 167 Gel-producing isolates, 59 (93.7%) and 94 (56.3%) isolates, respectively, possessed both the *asa1* and *esp* genes.

Transferability of asa1, cylA and aac(6')/aph(2") genes. To determine the transferability of *asa1*, *cylA* and *aac(6')/aph(2")* genes, mating experiments were performed. Of the 43 *E. faecalis* isolates possessing the 3 genes of *asa1*, *cylA* and *aac(6')/aph(2")*, 4 isolates were able to donate gentamicin resistance at a frequency of 10^{-4} to 10^{-1} per donor in broth matings. In filter matings, 7 of 43 and 28 of 43 isolates were able to donate the gentamicin resistance at a frequency of 10^{-4} to 10^{-1} and 10^{-8} to 10^{-5} per donor, respectively. The presence of *asa1*, *cylA* and *aac(6')/aph(2")* genes in transconjugants was confirmed by PCR assay. Of the transconjugants from the 35 *E. faecalis* isolates possessing *asa1*, *cylA* and *aac(6')/aph(2")*, 35 (100%) possessed the *aac(6')/aph(2")* gene, 33 (94.3%) possessed the *asa1* gene and 12 (34.3 %) possessed the *cylA* gene.

Clinical aspects on the isolation of E. faecalis in 4 groups based on the presence/absence of asa1 and esp genes. The 352 cases of UTI caused by *E. faecalis* consisted of 107 catheter-related (30.4%) and 245 catheter-unrelated (69.6%) cases, 202 polymicrobial (57.4%) and 150 monomicrobial (42.6 %) cases, and 60 febrile (17.0%) and 292 non-febrile (83.0%) cases. No statistically significant differences between biofilm-forming capacities and clinical background (catheter-related and catheter-unrelated cases, polymicrobial and monomicrobial cases, febrile and non-febrile cases) were found (Table 3). As shown in Fig. 4, both *asa1* and *esp* genes were carried by 20, 46, 79 and 85 isolates from patients with catheter-related monomicrobial UTI, catheter-related polymicrobial UTI, catheter-unrelated monomicrobial UTI and catheter-unrelated polymicrobial UTI, respectively. The *asa1* and/or *esp* genes were carried on 80 of 82 (97.6%) and 103 of 124 (83.1%) isolates from patients with catheter-related

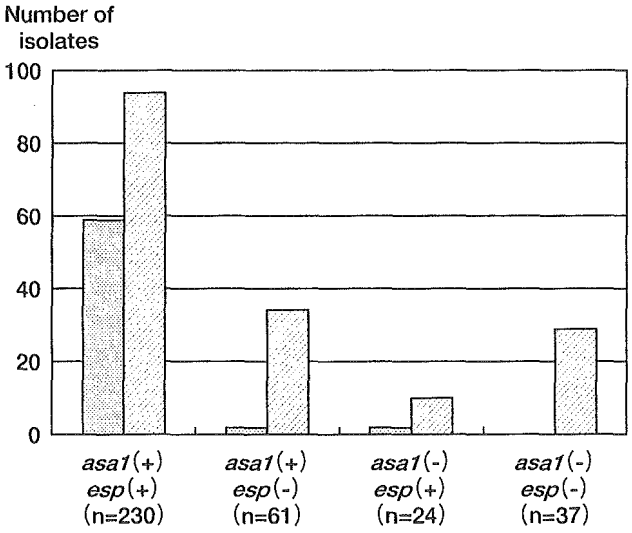

Fig. 3 Number of *E. faecalis* isolates producing hemolysin and gelatinase in 4 groups based on the presence/absence of *asa1* and *esp* genes.
Bars: ⁘, hemolysin; ▨, gelatinase

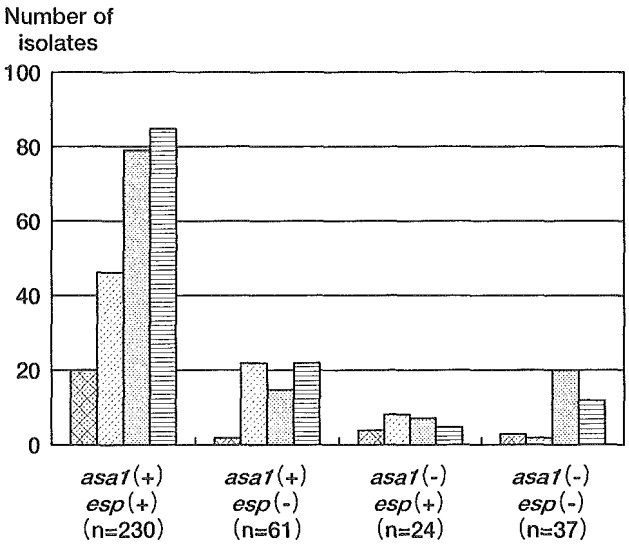

Fig. 4 Number of *E. faecalis* isolates in 4 groups based on the presence/absence of *asa1* and *esp* genes. Clinical aspects are shown by 4 categories.
Bars: ▩, catheter-related monomicrobial UTI
▨, catheter-related polymicrobial UTI
⁘, catheter-unrelated monomicrobial UTI
▤, catheter-unrelated polymicrobial UTI