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ปัจจัยความรุนแรงของเชื้อเอนเทอโรคอคคัส ฟีคาลิสชนิดดื้อยาเจนตามัยซินในระดับสูงที่แยกได้จากผู้ป่วยในโรงพยาบาล

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Virulence Factors in High-level Gentamicin Resistant *Enterococcus faecalis* Isolates from Hospitalized Patients

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หลักการและวัตถุประสงค์: เชื้อเอนเทอโรคอคคัส ฟีคาลิสชนิดดื้อยาเจนตามัยซินในระดับสูงเป็นปัญหาสำคัญของการติดเชื้อในโรงพยาบาลเนื่องจากการรักษาคนไข้ที่ติดเชื้อกลุ่มนี้จะทำได้ยาก การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจหาปัจจัยความรุนแรงของเชื้อเอนเทอโรคอคคัส ฟีคาลิสชนิดดื้อยาเจนตามัยซินในระดับสูงที่แยกได้จากผู้ป่วยในโรงพยาบาลจำนวน 45 ไอโซเลท

วิธีการศึกษา: การตรวจหาปัจจัยความรุนแรง ได้แก่ จีน *gelE, cylA* และ *esp* ทำโดยใช้เทคนิคพีซีอาร์ นอกจากนี้ยังได้ทำการตรวจหาการผลิต gelatinase, hemolysin และไบโอฟิลม์

ผลการศึกษา: เชื้อเอนเทอโรคอคคัส ฟีคาลิส ชนิดดื้อยาเจนตามัยซินในระดับสูง ตรวจพบจีนที่ควบคุมการสร้างปัจจัยความรุนแรงในการทำให้เกิดโรค ดังนี้ *gelE* พบ ร้อยละ 55.55; *cylA* พบ ร้อยละ 57.77; และ *esp* พบร้อยละ 86.66 การผลิต gelatinase พบร้อยละ 13.33 การผลิต hemolysin พบร้อยละ 62.23 และการสร้างไบโอฟิลม์พบ ร้อยละ 64.45

สรุป: เชื้อเอนเทอโรคอคคัส ฟีคาลิสชนิดดื้อยาเจนตามัยซินในระดับสูง มีปัจจัยความรุนแรงในการทำให้เกิดโรค ที่ควรให้ความสำคัญเพื่อควบคุมการแพร่กระจายของเชื้อดังกล่าว

Background and Objective: High-level gentamicin resistance (HLGR) in *Enterococcus faecalis* has become a serious problem in nosocomial infection due to the difficulty in the treatment of infected patients. The aim of the present study was to determine the incidence of potential virulence factors in 45 HLGR *E. faecalis* clinical isolates.

Methods: The virulence factor genes; *gelE, cylA* and *esp* were determined by PCR. The productions of gelatinase, hemolysin and biofilm were also investigated.

Results: The presence of virulence genes in the 45 HLGR *E. faecalis* isolates determined were 55.55%, 57.77% and 86.66% for *gel E, cyl A and esp*, respectivety. The productions of gelatinase, hemolysin and biofilm were found in 13.33%, 62.23% and 64.45% of *E. faecalis* isolates, repectively.

Conclusion: The HLGR *E. faecalis* clinical isolates possessed the virulence factors which have the potential to cause the disease. These findings suggest an effectively control strategy of these bacteria in hospitalized patients should be taken into consideration.

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Introduction

Enterococci are the commensal of gastrointestinal tract of humans and animals[1].They are also opportunistic pathogens that become a leading cause of nosocomial infections. *E. faecalis* accounts for the majority of nosocomial infections caused by enterococci[2]. They are intrinsically resistant to many antibiotics and are able to acquire resistance to antibiotics which caused failure of the treatment in hospitalized patients[3]. A combination of a cell wall active agent and an aminoglycoside is the treatment of choice for controlling enterococcal infection[4]. High level gentamicin resistant (HLGR) *E. faecalis* present problems in the treatment of infected patients, as synergy between penicillin and gentamicin is lost[5]. Previously, we have reported high level gentamicin resistance with multidrug resistance have emerged in 50% of *E. faecalis* clinical isolates in Srinagarind hospital[8]. This observation raises the question to be answered which virulence factors involve in the pathogenesis of these HLGR *E. faecalis*. A number of putative virulence factors have been described in *E. faecalis* to be associated with enterococcal infection. Among them, gelatinase (GelE), cytolysin, enterococcal surface protein (Esp) and biofilm formation have been studied most intensively[4, 6, 7, 19]. Gelatinase-producing *E. faecalis* isolates have been shown to be virulent in animal models and human infections[4]. Cytolysin is a hemolytic protein capable of lysing human, horse, and rabbit erythrocytes[4, 16]. Cytolysin-producing *E. faecalis* isolates have been shown to be associated with increased severity of infection[25]. Esp has been described to be involved in adhesion and colonization of the urinary tract[6]. Biofilm formation is an important process in initiating infection in the host[15, 18]. However, these virulence factors are still debated, because both infectious and non-infectious strains carrying the same virulence traits[19].

In this study, the incidences of *gelE*, *cylA*, *esp* genes and the production of gelatinase, hemolysin and biofilm were investigated in HLGR *E. faecalis* clinical isolates in order to understand the pathogenic potential of these isolates.

Methods

Bacterial strains

A total 45 clinical isolates of HLGR enterococci were obtained from clinical specimens submitted to the Clinical Microbiology Laboratory of Srinagarind Hospital, Khon Kaen, Thailand during June 2005 to 2007. Sources include urine 27 isolates, blood 3 isolates, pus 12 isolates and body fluid 3 isolates. All isolates were previously identified at species level by conventional methods[8]. The enterococci isolates were maintained in Brain Heart Infusion (BHI) broth containing 20% glycerol at -70 ºC until the time of analysis.

DNA preparation

The overnight culture (0.5 ml) was centrifuged at 13000 ×g for 5 minutes. The pellet was resuspended in TE buffer and heated at 95 ºC for 15 minutes in a heating block. The suspension was subsequently centrifuged at 13000 ×g for 15 minutes and the resulting supernatant containing the DNA was transferred to a sterile tube for PCR ampliflication of virulence genes[9].

Detection of virulence genes

The primers and their conditions used for amplification of the *esp*, *gelE* and *cylA* genes were previously described[10-12] (Table 1). PCR amplifications were performed in the GeneAmp PCR system 2400 (Perkin Elmer). The reaction were performed in a total volume of 25µl PCR mixture containing 2.5 µl of the bacterial lysate, 0.75 µl of 50 mM of $MgCl_2$, 0.5 µl of 10 µM of each primer, 0.5 µl of 1 U AmpliTaq DNA polymerase, 4 µl of 1.25 mM dNTPs, 2.5 µl of 10xPCR buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$; 0.01%w/v gelatin) and 13.75 µl of distilled water. PCR products were analysed on a 1.5% agarose gel in TBE buffer for 30 minutes at 100 V, stained with ethidium bromide, and visualized under UV light transilluminator (Quantum ST4). *E. faecalis* ATCC29212 and ENT011 were used as positive controls.

Table 1 Oligonucleotide primers used in this study

Genes	Primers	Sequence (5'-3')	Expected product (bp)	Amplification conditions
esp[10]	ESP14F	AGATTTCATCTTTGATTCTTGG	510	30 cycles of 94 ºC for 1 min
	ESP12R	AATTGATTCTTTAGCATCTGG		52 ºC for 1 min
				72 ºC for 1 min
gelE [11]	TE9	ACCCCGTATCATTGGTTT	419	30 cycles of 94 ºC for 30 sec
	TE10	ACGCATTGCTTTTCCATC		55 ºC for 30 sec
				72 ºC for 30 sec
cylA[12]	TE17	TGGATGATAGTGATAGGAAGT	517	30 cycles of 94 ºC for 45 sec
	TE18	TCTACAGTAAATCTTTCGTCA		57 ºC for 1 min
				72 ºC for 1 min

Gelatinase activity

Gelatinase activity was tested on BHI agar containing 3% gelatin[13]. After incubation at 37 °C for 48 hours, the plates were flooded with a saturated solution of ammonium sulphate. Gelatin precipitates and a transparent halo around cells appear in gelatinase producers. *E.faecalis* KU1857 was used as a positive control.

Hemolytic activity

Hemolytic activity was determined on BHI agar supplement with 5% human blood[14]. After incubation at 37 ºC for overnight under aerobic condition, hemolysin production was observed as β-hemolysis surrounding bacterial culture. *E. faecalis* ATCC29212 was used as a reference control.

Biofilm assay

E. faecalis were cultured in BHI broth containing 0.25% glucose in a flat-bottom sterile 96-well polystyrene microtitre plates (Nunclon). After incubation at 37°C for 24 h, the plate was washed 3 times with PBS and air dried in an inverted position at room temperature for 2 h. Two hundred microliters of 1% crystal violet were added. Each assay was performed in triplicate and repeated three times. Culture medium without any bacteria was used as a blank control. The biofilm formation was considered positive when an OD at 540 nm was > 0.1[15].

Results

The *gelE* gene was present in 25 of 45 *E. faecalis* isolates (55.55%). Twenty-six out of 45 HLGR *E. faecalis* isolates (57.77%) gave positive results for *cylA* gene and the *esp* gene was present in 86.66% of *E. faecalis* isolates (Figure 1). Distribution of phenotypic determinants; gelatinase, β-hemolysis and biofilm formation in high-level gentamicin resistance *E. faecalis* isolates is shown in Figure 2. Gelatinase activity was found in 6 of 45 *E. faecalis* isolates (13.33%). All *E. faecalis* isolates possessed *gelE* produced gelatinase activity, however 9 of 25 (36%) *E. faecalis* isolates possessed *gelE* without gelatinase activity. These results suggested that high-level gentamicin resistance *E. faecalis* isolates carrying *gelE* were not necessary for expression activity. Twenty-eight of 45 (62.22%) *E. faecalis* isolates exhibited β-hemolysis when these 26 isolates presence *cylA*. The ability to form biofilms of *E. faecalis* isolates was shown in Table 2. Twenty-nine out of 45 isolates (64.45%) of HLGR *E. faecalis* were biofilm producer.

Table 2 The ability to form biofilms of HLGR *E. faecalis* isolates

Categories	No. of *E. faecalis* isolates (%)
Non producer (OD< 0.1)	16 (35.55%)
Low producer (OD= 0.2-0.1)	26 (57.78%)
High producer (OD > 0.2)	3 (6.67%)
Total	45 (100%)

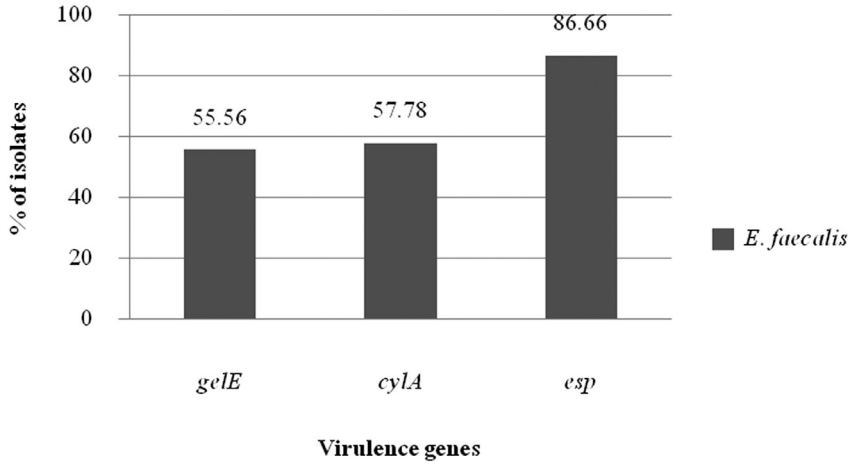

Figure 1 Distribution of virulence genes; *gelE*, *cylA* and *esp* in high-level gentamicin resistance *E. faecalis* isolates

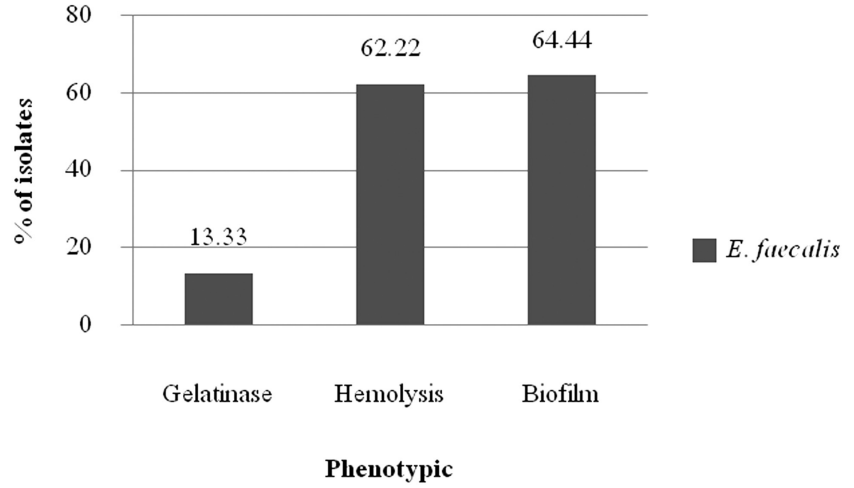

Figure 2 Distribution of phenotypic determinants; gelatinase, β-hemolysis and biofilm formation in high-level gentamicin resistance *E. faecalis* isolates

Discussion

High-level gentamicin resistant (HLGR) *E. faecalis* isolates have become a serious problem in the treatment of infected patients. It is predictive of the loss of the synergy between gentamicin and ampicillin. This makes the treatment of serious enterococcal infection difficult[1]. Several virulence factors, such as GelE, enterococcal surface protein (Esp), cytolysin, and biofilm formation are possibly associated with the colonization and pathogenesis of enterococci[4, 6, 7].

The distribution of virulence factors including *gelE*, *esp*, *cylA*, hemolysin, gelatinase and biofilm production in HLGR *E. faecalis* clinical isolates were studied. Gelatinase is an enzyme capable of hydrolyzing gelatin and other peptides. It may also cause direct or indirect damage to host connective tissue or tissue proteins[4]. In this study, the production of gelatinase was found in 13.33% *E. faecalis* isolates. This finding differs from the previous studies which showed 55% and 68% of *E. faecalis* from blood culture produced gelatinase[16, 17]. However, *gelE* was present in 55.55% of *E. faecalis* isolates indicating that some *gelE* could not express for gelatinase production. This may be due to the presence of silent *gel E* genes[11] or the absence of subunits required for *gelE* expression[13]. The expression of *gelE* are required the *fsr* operon which may be lost during freezing in laboratory conditions[13, 24].

Cytolysin is a bacterial toxin expressed by some strains of *E. faecalis* which are required for bactericidal and hemolytic activity[4]. In this study, the *cylA* gene was found in 57.77% *E. faecalis* but cytolysin production was found in 62.23% *E. faecalis*. The *cylA* gene was not found in 2 of 28 isolates which produced hemolysin activity. This suggests that either other genes may have encoded hemolytic activity or that the primer used in our studies was too specific to cover all the *cylA* genes[26].

The *esp* was detected in 86.66% HLGR *E. faecalis* isolates. These results suggest that *esp* gene may associated with pathogenesis of HLGR *E. faecalis*. It has been shown previously that *esp* gene enriched in infection derived isolates[12]. The frequency of biofilm formation appeared in 64.45% *E. faecalis*. Some reports have shown that biofilm formation capacity is restricted to strains harbouring the *esp* gene[18, 20, 21]. However, other authors have demonstrated that biofilm formation may exist independently from Esp protein[22, 23]. We found that *E. faecalis* with both *esp*-positive and *esp*-negative isolates could form biofilm, suggesting that independent environment conditions may contribute to biofilm formation.

In conclusion, high-level gentamicin resistance *E. faecalis* isolates from hospitalized patients harbored potential virulence traits which make them causing nosocomial infections. These findings suggest the importance to control the spreading of these bacteria.

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