An active surveillance study of vancomycin-resistant Enterococcus in Queen Elizabeth Hospital, Hong Kong

Objective. To assess the rate of faecal vancomycin-resistant Enterococcus colonisation in high-risk patients in a regional hospital.

Design. Prospective observational surveillance study.

Setting. Queen Elizabeth Hospital, Hong Kong.

Patients. From September 2001 to December 2002, stool samples from patients in the intensive care unit and patients in whom Clostridium difficile testing was requested were used for study using a broth enrichment method.

Main outcome measures. Number of faecal vancomycin-resistant Enterococcus colonisation.

Results. A total of 2414 cultures from 1792 patients were tested for vancomycin-resistant Enterococcus using a broth enrichment method. Only one (0.06%) patient was found to harbour a vancomycin-resistant Enterococcus faecalis in the gastro-intestinal tract. Surveillance cultures from contacts of the case revealed another six with vancomycin-resistant Enterococcus faecalis. Vancomycin-resistant Enterococcus faecalis was also later reported from a clinical specimen (catheterized urine) of another patient. They were all epidemiologically linked to the index case. Mean inhibitory concentrations of vancomycin and teicoplanin were determined to be higher than 256 and 0.5 µg/mL, respectively by E-test for all the vancomycin-resistant Enterococcus isolates. Polymerase chain reaction analysis confirmed the presence of vanB genes and the result was in line with the phenotype. Pulsed-field gel electrophoresis confirmed a monoclonal vancomycin-resistant Enterococcus outbreak. Strict infection control measures recommended by the Centers for Disease Control and Prevention were followed and the outbreak was successfully controlled.

Conclusion. Vancomycin-resistant Enterococcus colonisation is rare, but present among high-risk patients in our hospital. A routine surveillance programme should be implemented that will enable early case detection and prompt initiation of infection control measures to prevent the emergence of an endemic situation.

Key words: Clostridium infections; Cross infection; Enterococcus; Feces/microbiology; Vancomycin resistance

關鍵詞：梭狀桿菌感染；交叉感染；腸球菌；糞便／微生物學；萬古霉素的抗藥性
Introduction

Vancomycin-resistant Enterococcus (VRE) was first detected in Britain in 1986 and reported in 1988, closely followed by similar reports from other European countries and the United States. Recent data from the Centers for Disease Control and Prevention National Nosocomial Infections Surveillance System indicate that in 2001, 20% to 30% of nosocomial enterococcal infections were resistant to vancomycin. At present, more than 20% of enterococci isolated from intensive care units (ICUs) exhibit vancomycin resistance.

The rapid emergence of VRE in the United States has been attributed to the intensive clinical use of vancomycin in both parenteral and oral forms in that country on a background of high-level usage of cephalosporins, that promote enterococcal superinfection. In Europe, investigators have postulated an additional role for the use of the glycopeptide avoparcin as a growth promoter in intensive animal industries, resulting in colonisation with vanA Enterococcus faecium and subsequent transmission to humans via the food chain. In Hong Kong, the first vancomycin-resistant E faecium case was detected in our hospital in 1997 in a patient returning from the United States. Subsequent sporadic cases of colonisation were later identified in clinical specimens from several other hospitals. The true prevalence of VRE in Hong Kong is unknown due to the lack of a standardised, active surveillance programme.

To determine the prevalence of VRE colonisation in high-risk groups, samples from ICU patients and other hospitalised patients requiring Clostridium difficile cytotoxin assay were studied.

Patients and methods

Study design

Queen Elizabeth Hospital is a 1850-bed, acute-care, teaching tertiary centre. A prospective four-point surveillance was carried out quarterly between 1 September 2001 and 31 December 2002 in a combined medical and surgical ICU with a total of 22 beds and 95% occupancy rate. All patients had stool samples obtained on admission to the ICU and twice weekly while in the unit. If a stool sample was not available, a rectal swab with visible faecal component was collected. The surveillance cultures were collected by nursing staff according to a prescribed procedure. Other stool samples that required C difficile cytotoxin testing were also tested for VRE throughout the study period.

Microbiology

All specimens were processed on the same day as collection. At the weekend or on public holidays, samples were stored at 4°C and processed on the next working day. A standard inoculation and incubation procedure was followed. A rectal swab or 1 g of stool sample was directly inoculated into 5 mL of D-Enterococcus broth (BBL; BD, New Jersey, US) supplemented with 6-µg vancomycin per mL. The D-Enterococcus broth was incubated at 35°C for 72 hours, and inspected daily for signs of colour change. If the broth turned black, it was subcultured onto D-Enterococcus agar (BBL) supplemented with 6-µg vancomycin per mL and onto 5% horse blood agar separately and incubated at 35°C overnight.

Identification

The suspected enterococcal colonies were identified at the genus level using cellular morphology, reaction to gram staining, bile esculin hydrolysis, catalase and pyrrolidonyl arylamidase (Rosco Diagnostica, Taastrup, Denmark) activity. API 20 STREP system (BioMérieux Vitek Inc, Hazelwood [MO], US) was applied to identify the isolates to species level. To confirm vanC enterococci, supplementary tests were conducted for motility by stab inoculation into motility medium. Pigmentation production was determined by taking a sweep from an area of heavy
growth on brain heart infusion agar using a cotton swab and examining it for a bright yellow colour.

Susceptibility testing
Susceptibility testing was carried out using a standard disk diffusion for ampicillin (10 µg), tetracycline (30 µg), nitrofurantoin (300 µg), erythromycin (15 µg), rifampicin (5 µg), levofloxacin (5 µg), linezolid (30 µg), vancomycin (30 µg; BioMérieux Vitek Inc), and teicoplanin (30 µg; BioMérieux Vitek Inc) on Muller Hinton agar with 24 hours’ incubation at 35°C. The criteria recommended by the National Committee for Clinical Laboratory Standards were used. The nitrocefin test was carried out to detect the presence of the β-lactamase. The minimum inhibitory concentrations (MICs) for vancomycin and teicoplanin were determined by the E-test method (AB Biodisk, Solna, Sweden) according to the manufacturer’s instructions.

Contact tracing
If surveillance resulted in detection of a patient with VRE, the Hospital Infection Control Team was informed immediately and infection control measures, as recommended by the Hospital Infection Control Committee, were implemented. A line listing of contact cases was recorded and surveillance cultures from contact cases were taken to assess the degree of spread. A contact case was defined as a patient who had been in close proximity to the source patient, such as in the same ward at the time the index case was detected. Rectal swabs containing visible faecal components were collected by the infection control nurses. The collected sample was directly inoculated onto D-Enterococcosel broth supplemented with 24 µg/mL of vancomycin and incubated at 45°C for 72 hours. They were inspected daily for any change in colour. If the broth turned black, it was subcultured onto the Enterococcosel agar plate supplemented with 24 µg/mL of vancomycin and incubated at 45°C overnight. Further identifications, antibiotic susceptibility testing, and vancomycin MIC determination, were performed as described in the methodology. Other sites of VRE patients were also screened to assess the degree of colonisation: they included the perineal, axillary, and umbilical area. A midstream specimen of urine was also obtained. A designated area of about 10 cm² was swabbed using cotton swabs and directly inoculated into 5 mL of D-Enterococcosel broth supplemented with 24 µg/mL of vancomycin and incubated at 45°C for 72 hours. The standard incubation procedure was then followed.

Staff screening
Health care workers who had contact with VRE-positive patients were screened to detect VRE carriage. After thoroughly washing their hands, staff were instructed to place their hands into a board-based plastic container that contained 10 mL of Enterococcosel broth supplemented with 24 µg/mL of vancomycin. The standard incubation procedure was then followed.

Environmental screening
Surfaces in VRE patients’ environment were sampled to assess the degree of environmental contamination: bed rails, bedside tables, bedside chair, medical charts, bed linen, doorknobs, taps, blood pressure cuff, sink, and bedpans. Samples were obtained by swabbing 10 cm² of the designated surface using premoistened cotton swabs. The cotton swabs were directly inoculated into 5 mL of Enterococcosel broth supplemented with 24 µg/mL of vancomycin and incubated at 45°C for 72 hours. The standard incubation procedure was then followed.

Detection of van genes by polymerase chain reaction
The VRE (vancomycin MIC, ≥32 µg/mL) strains were sent to the reference laboratory, Public Health Laboratory Centre, Department of Health, for molecular testing. Polymerase chain reaction (PCR) was not performed for the vancomycin-intermediate Enterococcus (vancomycin MIC, 8-24 µg/mL) isolates. DNA extraction by DNAzol Reagent (Invitrogen; Life technologies, California, US) was performed according to the manufacturer’s instructions. The

<table>
<thead>
<tr>
<th>Primer specificity</th>
<th>Primer pair sequences</th>
<th>Size of PCR products (bp)</th>
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</thead>
<tbody>
<tr>
<td>vanA (Enterococcus faecium, MIB40)</td>
<td>5'-GCTATTCAGCTGTACTC-3' 5'-CAGGGCCCATCATACGG-3'</td>
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<tr>
<td>vanB (Enterococcus faecalis, ATCC 51299)</td>
<td>5'-CATACGGCCCGAGCTTCACC-3' 5'-GATCGGAAGATACCCGCT-3'</td>
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<tr>
<td>vanC1 (Enterococcus gallinarum)</td>
<td>5'-GGTATCAAGAAACCTGCT-3'</td>
<td>822</td>
</tr>
<tr>
<td>vanC2 (Enterococcus casseliflavus)</td>
<td>5'-CTTCGCGATCATAGCT-3' 5'-CTCTCACGATTCCTTG-3' 5'-CGAGCAAAGCCTTTAAG-3'</td>
<td>439</td>
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</tbody>
</table>

Table 1. Polymerase chain reaction (PCR) primers for detection of vancomycin-resistant Enterococcus

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four primer sets shown in Table 1 were added to the reaction mixtures. Polymerase chain reaction assay was performed in a total volume of 50 µL containing 2 mM of MgCl₂; 0.5 pmol of each of the vanA, vanB, vanC1, and vanC2 primers; 10 µM of each of deoxynucleotide triphosphate (dATP, dGTP, dTTP, dCTP); and 0.025 U of AmpliTaq. DNA amplification was carried out with the following thermal cycling profile. Initial denaturation at 95°C for 10 min, 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min), and a final extension at 72°C for 1 min in an Applied BioSystem 9700 (Applied BioSystem, Foster City, US). Polymerase chain reaction products were analysed on a 1% agarose gel (Sigma; Chemical Co, St Louis, US) with 1X Tri-borate-EDTA buffer. A 100 bp DNA ladder (Invitrogen) was used as the molecular size marker. Gels were stained with ethidium bromide and photographed under ultraviolet light. Amplification of vanA, vanB, vanC1, and vanC2 targets produced distinct bands that corresponded to their respective molecular sizes and were easily recognised. The PCR assay was carried out with a negative control containing all of the reagents without a DNA template.

**Pulsed-field gel electrophoresis**

Clonality of VRE was determined by pulsed-field gel electrophoresis (PFGE) of SmaI restriction digests of genomic DNA with a Biorad Genepath System (Biorad Laboratories, Hercules, California, US) according to the manufacturer’s instructions. Electrophoresis was performed and the gel was stained with ethidium bromide and photographed under ultraviolet light. Amplification of vanA, vanB, vanC1, and vanC2 targets produced distinct bands that corresponded to their respective molecular sizes and were easily recognised. The PCR assay was carried out with a negative control containing all of the reagents without a DNA template.

**Medical review**

Demographic data and risk factors for VRE acquisition were analysed by reviewing patients’ medical records. Parameters such as age, sex, underlying disease, length of hospitalisation, enteral feeding, vascular access, antibiotic exposure (including cephalosporins, anti-anaerobes, and vancomycin), presence of a urinary catheter, mechanical ventilation, history of diarrhoea or surgery, site of VRE isolation, and outcome were assessed.

**Results**

A total of 2414 surveillance cultures from 1792 patients were screened for VRE: 752 specimens were collected from 343 patients from ICU, and 1662 stool samples were collected from 1449 patients for whom C difficile testing was requested. This represented 19.5% (343/1757) of the annual ICU admission, and 0.82% (1449/176 608) of all hospital admissions. One patient with a stool sample submitted for C difficile testing was found to be VRE positive. No VRE was isolated from patients in ICU. The overall prevalence of VRE colonisation was 0.06% (1/1792). The MICs of vancomycin and teicoplanin were determined to be higher than 256 and 0.5 µg/mL, respectively by E-test. Vancomycin-intermediate enterococci (MIC: vancomycin 8-24 µg/mL and sensitive to teicoplanin) were found to be carried by 207 (11.6%) of 1792 patients. Further verification of these isolates using API 20 STREP (BioMérieux Vitek Inc) augmented with the supplementary tests (motility and yellow pigmentation production) identified 203 (11.3%, 203/1792) and four (0.2%, 4/1792) as *Enterococcus gallinarum* and *Enterococcus faecalis*, respectively. A total of seven
Vancomycin-resistant Enterococcus

VRE-positive cases were detected among 47 contact patients. Six of them were identified according to the method specified under the contact tracing section. The remaining patient was culture-positive for VRE in his clinical sample of catheterized urine. The patient was subsequently found to have contact with the index case. The reason for missing out this patient in the original contact tracing protocol was that he had already been transferred to another hospital prior to detection of the index case. These seven VRE cases were thus epidemiologically linked to the index case, as they had all been admitted to the same ward. A total of eight vancomycin-resistant E faecalis were recovered with similar biochemical and susceptibility profiles (Table 3). A total of 265 environmental swabs were sampled. Twenty-one (7.9%) VRE isolates were recovered from environmental surfaces: all were vanB type E faecalis with similar biochemical and susceptibility profiles to the VRE strains isolated from patients. Thirty-four health care workers were screened for VRE, including six medical staff, 15 nursing staff, eight physiotherapists, and five health care assistants. They were all VRE negative.

Table 3. Susceptibility profiles of vanB Enterococcus faecalis isolates*

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>VRE-1</th>
<th>VRE-2</th>
<th>VRE-3</th>
<th>VRE-4</th>
<th>VRE-5</th>
<th>VRE-6</th>
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<td>Vancomycin (30 µg)</td>
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<td>Vancomycin MIC (µg/mL) by E-test</td>
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<td>Teicoplanin MIC (µg/mL) by E-test</td>
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* VRE denotes vancomycin-resistant Enterococcus; MIC minimum inhibitory concentration, S sensitive, I intermediate, and R resistant; this was interpreted according to the recommendations of NCCLS criteria

Twelve selected VRE isolates (eight patient isolates and four environmental strains) were confirmed by PCR to carry vanB genes. The results were in line with the phenotype by MIC determination.

The same sets of VRE isolates were analysed by PFGE. Analysis of the banding patterns showed identical banding patterns in six VRE strains, while another six strains had 1 to 2 bands difference and were classified as genetically related (Fig).

Demographic data and associated risk factors from the eight VRE cases were identified (Table 4) after review of clinical records. Risk factors for acquisition of VRE, such as antibiotic exposure (cephalosporin, anti-anaerobes, and vancomycin), prolonged hospitalisation (mean, 132 days), and presence of vascular access occurred in all patients. Other risk factors, such as enteral feeding, presence of urinary catheter, and mechanical ventilation were also present in most patients. Based on the clinical
All patients colonised with VRE were isolated in a single room with strict contact precautions until discharge or eradication of VRE. Rooms were cleansed using chlorax twice daily and terminal cleansing performed when the patient was transferred out. Re-admission of a new case was permitted only if environmental screening for VRE after terminal cleansing was negative. The index case remained VRE-positive on discharge. Vancomycin-resistant Enterococcus was eradicated from three of the contact cases within 3 months of initial detection (defined by having three consecutive negative cultures from all previously colonised sites taken over a 3-week period). The remaining four were transferred to convalescent wards in other centres and followed up by infection control nurses there.

**Discussion**

This study was undertaken to determine the prevalence and the genetic properties of VRE and to assess determinants associated with VRE carriage in a selected high-risk category of hospitalised patients in ICUs. Stools samples submitted for *C. difficile* cytotoxin testing have been included in the surveillance programme. Adopting this screening strategy...
has several benefits. First, antibiotic exposure plays a role in the development of *C difficile*: the epidemiology is similar to that of VRE disease. Screening stool samples submitted for *C difficile* cytotoxin testing is thus an effective way to detect gastrointestinal tract colonisation with VRE. Second, patients with diarrhoea are more likely to shed VRE into their environment, leading to the colonisation of other patients in the proximity.

There is no uniformly accepted screening method for VRE. Numerous types of commercially available and in-house–prepared selective agar and broth formulations have been used. Although solid media are often used in screening studies, culture on VRE selective medium after a broth enrichment step is the most sensitive method for detection. A concentration of 6 µg of vancomycin per mL has been shown to be reliable for the detection of enterococcal strains with low-level vancomycin resistance. The quantitative analysis conducted in this study (Appendix 1) concurred with the findings of published studies. The Enterococcosel broth has a detection threshold of 1 colony-forming unit (CFU) per mL in faeces, while direct plating on Enterococcosel agar plate could only detect VRE at a concentration of at least 100 CFU per mL of faeces during this experiment. Therefore, the broth enrichment was incorporated as an essential step in this study of detection protocol (Appendix 2). Enrichment of cultures nonetheless entails a greater amount of work and may not be readily feasible for routine surveillance in a hospital setting with limited resources and reduced manpower.

Among the enterococci, *E gallinarum* and *Enterococcus casseliflavus* are relatively infrequent causes of human infection. vanC1 and vanC2 gene clusters are responsible for the intrinsic low-level vancomycin resistance in these groups of organisms that have little impact on hospital infection control since the vanC genes are not transferable. Most nosocomial infections are caused by *E faecium* and *E faecalis* that may carry the vanA and vanB genes on the conjugative transposon and may be transferred among and/or across the species. These observations emphasise the need for accurate identification of enterococcal species and detection of acquired or intrinsic vancomycin resistance. Some of the isolates in this study could not be identified to species level by commercial kits, and required further supplementary tests, for example, motility at 30°C and production of yellow pigmentation, to verify isolates as *E gallinarum*. Nonetheless differentiation among *E faecium*, *E gallinarum*, and *E casseliflavus* based on these physiological tests is not totally reliable. Minimum inhibitory concentration alone is insufficient to determine the genetic status of VRE, especially for the low-level VRE. Polymerase chain reaction assay provides an alternative for rapid and accurate detection and identification of low-level resistant enterococci, especially during outbreaks, but its cost-effectiveness for routine surveillance in low-prevalence countries remains unproven.

Only one vancomycin-resistant *E faecalis* was isolated from the stool sample submitted for *C difficile* cytotoxin assay. The patient was a 31-year-old female who presented with fever, headache, and dizziness and was admitted to a neurosurgical ward for investigation in early February 2002. Magnetic resonance imaging findings suggested meningioma and obstructive hydrocephalus. Emergency craniotomy was performed and a tracheostomy inserted postoperatively. She was fed enterally and had an indwelling urethral catheter. Multiple antibiotics were prescribed, including cephalosporins, anti-anaerobes, and vancomycin for over 7 days. She had severe diarrhoea in early April. Stool collected for *C difficile* cytotoxin assay was positive for *C difficile* cytotoxin and VRE. Further active surveillance cultures identified six more ‘occult’ VRE cases that originated from the neurosurgical wards. Positive findings from other body sites (perineum, axilla, and urinary tract) and environmental surfaces (bedside tables, bed rails, walking frame, sink, and medical chat) confirmed a high colonisation density and environmental shedding. This may have facilitated the spread of VRE to other patients in close proximity by direct or indirect transmission through the hands of health care workers or inanimate objects. This could not be proven as the staff were screened negative for hand carriage. It may be that an increased awareness of staff of the study increased compliance with good handwashing practices and increased the use of alcoholic handrubs after the ‘outbreak’. Neither rectal swabs nor stool samples from health care workers were screened for VRE since VRE stool colonisation appeared to be rare among them. Isolation and contact precaution measures were initiated promptly as recommended by the Hospital Infection Control Practices Advisory Committee. Antibiotic usage was reviewed and recommendations were made. Multi-disciplinary efforts ensured further spread was halted and no more VRE cases were reported.

The results of this study based on the molecular
typing suggest a monoclonal outbreak of vanB type E faecalis among the neurosurgical patients. Cross-infection among patients was the most likely route of transmission. The exact chain of events nonetheless could not be ascertained, nor the significance of the environment in the outbreak. When VRE emerges and infection control measures are not implemented, it can spread readily among patients due to its intrinsic properties, such as gastro-intestinal and skin colonisation and prolonged survival in the environment. The source is difficult to identify retrospectively: it is not known if the first VRE case identified during the surveillance study was the index case or a case infected by another index case. The original VRE may have become established either by de-novo selection of resistant van gene clusters of E faecalis in the gastro-intestinal tract due to antibiotic pressure during prolonged hospitalisation or community-acquired gastro-intestinal tract colonisation prior to hospitalisation.

The prevalence of VRE colonisation in high-risk patients was 0.06%, which is very low compared with other studies.5,16 Although other high-risk groups, such as those from renal, haematology-oncology, or bone marrow and solid-organ transplant units were not recruited during this surveillance, stools submitted for C difficile testing did originate from some of these patients. Underestimation of the true prevalence is thus minimised. A routine VRE surveillance programme to enable early case detection and prompt infection control implementation is strongly recommended for endemic settings. The cost-effectiveness of screening for VRE in a low-prevalence environment, such as the study hospital, is unknown.17 Nevertheless, a focused surveillance programme is critically important for a selected category of high-risk patients. This is because approximately 95% of VRE-colonised patients18 and 87% of VRE cases in this study had no clinically positive microbiological culture. Infection control measures would be difficult to implement in the absence of knowledge of an index case. In addition, the genetic transfer of vancomycin-resistant genes to methicillin-resistant Staphylococcus aureus present in VRE carriers19 poses the threat of a more virulent-resistant organism emerging, thus limiting therapeutic choices and increasing morbidity and mortality.20

**Conclusion**

Vancomycin-resistant Enterococcus colonisation is rare but present among hospitalised patients in our locality. Routine continuous surveillance targeted at high-risk patients, preferably those with samples sent for C difficile cytotoxin testing, should be in place to prevent the situation from worsening.

**Appendices**

Additional material related to this article can be found on the HKMJ website. Please go to <http://www.hkmj.org.hk>, search for the appropriate article, and click on Full Article in PDF following the title.

**Acknowledgements**

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**References**

Vancomycin-resistant Enterococcus


Appendix 1

In a cross-sectional study, Ieven et al.12 showed that broth enrichment culture detected an additional 46.5% of vancomycin-resistant Enterococcus (VRE) cases compared with direct agar plating. To verify these findings, we conducted a simple study before the surveillance programme.

Procedures

Faecal suspension preparation
1 g of faeces of clinical sample was weighed and mixed in 10 mL of physiological saline to produce 0.1 g/mL of faecal suspension.

Bacterial suspension
Cell suspension at a density equivalent to a McFarland standard of 0.5 (108 colony-forming unit [CFU]/mL) was prepared from vancomycin-resistant Enterococcus faecium, minimum inhibitory concentration (MIC) 24 µg/mL (external QC from RCPA 2000: 2:IB). Serial 10-fold dilutions were then made with normal saline to produce VRE concentrations of 107, 106, 105, 104, 103, 102 CFU/mL. Accurate colony counts were determined by inoculation of the serial bacterial concentrations onto horse blood agar plates that were incubated overnight at 35°C.

Vancomycin-resistant Enterococcus–faecal suspension
10 mL of the bacterial suspension was mixed with 1 mL of faecal suspension (see above) to achieve final VRE concentrations as follows: 105, 104, 103, 102, 10, 1 CFU/mL of faecal suspension. Aliquots were taken from each of the VRE-faecal dilution tubes and 1 mL inoculated into Enterococcosel broth and 10 mL plated onto Enterococcosel agar plates followed by overnight incubation at 35°C. Inoculation of the faecal suspension without VRE served as a negative control.

Results

The broth enrichment method can detect VRE presence at levels of 1 CFU/mL of stool, while direct agar plating requires much higher levels of 100 CFU/mL of stool. No growth was detected in the negative control. Count plating confirmed accurate bacterial counts in the serial dilutions. The colonies detected were confirmed to be E faecium with MIC 24 µg/mL by API 20 STREP and E-test.

Conclusion

The broth enrichment method is considerably more sensitive than the agar plating method for detecting VRE in stool samples. We incorporated the broth enrichment method as an essential step in the VRE detection protocol.

Appendix 2

1 g/mL of faecal suspension, or rectal swabs was added to 5 mL of Enterococcosel broth with 6 µg/mL vancomycin

No growth after 3 days, broth discarded

Incubate at 35°C for 3 days and daily inspection for black discolouration

If it turns black, subculture onto:
1. Enterococcosel agar supplemented with 6 µg/mL vancomycin, and
2. Blood agar with vancomycin (30 µg) disk put on the first quadrant

Incubate at 35°C overnight

1. Identify different morphologies, and make gram stain
2. If there is gram-positive coccus, perform pyrrolidonyl-beta-naphthylamide (PYR) test
3. If PYR test is positive, plate out on blood agar ad perform vancomycin E-test using brain heart infusion agar, and disk diffusion susceptibility testing using Muller Hinton agar

Incubate the E-test for full 48 hours and disk diffusion test for 24 hours at 35°C

If minimum inhibitory concentration >4 µg/mL, perform API 20 STREP on each isolate

If identification is up to genus level only, perform supplementary test,
1. Motility by stab inoculation into motility medium at 30°C
2. Pigment production by making a sweep over the heavy growth on the brain heart infusion agar