ORIGINAL ARTICLE

Survivability of vancomycin resistant enterococci and fitness cost of vancomycin resistance acquisition

A A Ramadhan, E Hegedus

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See end of article for authors' affiliations

Correspondence to: Mr A A Ramadhan, School of Biomedical Sciences, Faculty of Health Sciences, Cumberland Campus, PO Box 170, Lidcombe, NSW 1825, Australia; Abdull20@yahoo.com

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Aims: To investigate the survivability of vancomycin resistant enterococci (VRE) under dry starvation conditions and the fitness cost of vancomycin resistance.

Methods: VRE colonies on cotton swabs were incubated at room temperature in a sterile box and cultured weekly until cultures no longer showed growth. Negative swabs inoculated into brain heart infusion (BHI) broth were subcultured to blood agar after 24, 48, and 72 hours of incubation to resuscitate viable but non-culturable cells. Stability of the vancomycin resistance determinant and of the DNA fingerprint pattern was determined by multiplex polymerase chain reaction (PCR) and repetitive PCR, respectively. Tests for fitness cost were carried out on the same VRE isolates and 28 hospital vancomycin sensitive enterococci (VSE) isolates by incubation and measurement of optical density using a microplate reader and comparing maximum growth rate and lag phase duration between VRE and VSE, using independent samples *t* tests.

Results: Mean maximum time of recovery by primary culture was 8.5 weeks for *Enterococcus faecalis* VRE and 21.8 weeks for *E faecium* VRE. Two of two *E faecalis* isolates were resuscitated after 24 hours in BHI broth, and two of five *E faecium* isolates after 72 hours. No fitness cost of vancomycin resistance was demonstrated.

Conclusions: VRE can survive for prolonged periods in a dry starvation state, retaining their genetic complement, including vancomycin resistance determinants, and show little or no fitness cost of vancomycin resistance. Thus, the rate of entry required for VRE to become, and remain, endemic in the community is relatively small.

Vancomycin resistant enterococci (VRE) have emerged as important nosocomial pathogens since first described in 1987, and there is concern that they may be, or become, endemic in the non-hospital setting, both in human and animal carriers and in the general environment. This would depend on the rate of introduction, the survivability of the organisms in the environment, and the ability of the organisms to compete with vancomycin sensitive enterococci (VSE).

> ''Vancomycin resistant enterococci have been shown to be capable of surviving on dry surfaces in hospitals for up to four months''

More often than not, the first factor in the persistence of an organism in the environment will be an ability to survive under virtual starvation conditions. VRE have been shown to be capable of surviving on dry surfaces in hospitals for up to four months.[1 2] However, there are no studies of the survivability of community isolates of VRE under similar circumstances.

Another means by which VRE might survive in the environment is as viable but non-culturable cells (VBNC). A research group at the University of Verona, Italy, has been active in investigating the VBNC state in enterococcus. This group found that *Enterococcus faeecalis*, but not *E faecium*, readily entered a VBNC state, and that vancomycin resistance was maintained in enterococci in the VBNC state and after division was resumed.[3–9]

No studies have been reported on the production of VBNC under conditions of dry starvation.

Ultimately, a capacity for survival for extended periods under starvation conditions is not sufficient for persistence in the environment: the organism must also be able to grow and compete with other organisms.

A large number of studies have shown that resistant bacteria often have lower growth rates than their susceptible competitors in the absence of antibiotics. However, it has also been shown that the costs of antibiotic resistance can evolve and tend to be reduced over time by natural selection.[10]

Our study set out to investigate the survivability (including possible production of VBNC) of VRE under dry starvation conditions and the fitness cost of vancomycin resistance, as an indication of the likelihood of VRE becoming, or remaining, endemic in the general community.

METHODS

VRE survivability

Growth and starvation of cells

Control strains of vanA *E faecium* and vanB *E faecalis* and four strains of vanB *E faecium* and one strain of vanB *E faecalis* isolated from faeces from children in a survey of childcare centres in Sydney, Australia were cultured on blood agar plates at 37℃ for 24 hours. Five separate colonies of each isolate were then picked up on cotton swabs, inserted into a container, and incubated at room temperature in a sterile box. The only source of nutrients was thus dead cells on the swab.

Recovery of starved cells

On a weekly basis, the swabs were inoculated on to blood agar plates and incubated at 37℃ for 48 hours. This was repeated until cultures no longer showed growth or for a minimum of eight weeks.

Negative swabs were inoculated into brain heart infusion (BHI) broth and incubated at 37℃ for 72 hours to resuscitate

Abbreviations: BHI, brain heart infusion; PCR, polymerase chain reaction; VBNC, viable but non-culturable cells; VRE, vancomycin resistant enterococci; VSE, vancomycin sensitive enterococci

Table 1 Survivability of VRE on dry cotton swabs incubated at room temperature and results of attempted resuscitation of possible VBNC in BHI broth at 37°C

Strain	Maximum time of recovery (weeks)	Attempted resuscitation
Control vanA *E faecium*	23	Negative after 72 hours
Control vanB *E faecalis*	9	Positive after 24 hours
vanB *E faecium* (code 1)	19	Negative after 72 hours
vanB *E faecium* (code 2)	21	Negative after 72 hours
vanB *E faecium* (code 3)	23	Positive only after 72 hours
vanB *E faecium* (code 4)	23	Positive only after 72 hours
vanB *E faecalis* (code 5)	8	Positive after 24 hours

BHI, blood heart infusion; VBNC, viable but non-culturable cells; VRE, vancomycin resistant enterococci.

any VBNC. After 24, 48, and 72 hours of incubation, an inoculum was transferred on to blood agar to check for growth. Isolates showing growth were assumed to have entered a VBNC state, and the time of entering this state was calculated from the last week showing positive culture plates on direct culture.

After resuscitation, the stability of the vancomycin resistance determinant and of the DNA fingerprint pattern was determined by multiplex polymerase chain reaction (PCR) and repetitive PCR, respectively.

Fitness cost of vancomycin resistance

Growth kinetics test

The VRE strains listed above were compared for maximum growth rate and length of lag phase with 28 clinical isolates from a Sydney hospital. All isolates were inoculated on to BHI agar plates and incubated overnight at 37°C. One colony of each isolate was suspended in phosphate buffered saline and diluted to an optical density of 0.1 at 595 nm using a microplate reader (Bio-Rad, Philadelphia, Pennsylvania, USA). A 1 µl aliquot of each suspension was then added to 100 µl of BHI broth in each of three wells of a 96 well plate.

Using the microplate reader, the cultures were incubated at 37°C, with shaking every 15 minutes. Optical density readings at 595 nm were taken from each well every 10 minutes over 10 hours and plotted against time. The maximum value of the change in optical density each minute over any 100 minute period was taken to indicate the maximum growth rate for each cell, whereas the lag phase duration was taken to be the beginning of the maximum growth rate.

Statistical methods

Mean maximum growth rates and lag phase duration for VRE and VSE were compared by the independent samples *t* test, using SPSS software version 11.5.

RESULTS

Survivability

Table 1 shows the results of the survivability experiment.

A gradual decline in total cell numbers started at week 4 for *E faecalis* strains, but not until week 9 for *E faecium* strains. The mean maximum time of recovery by plating was 8.5 weeks for the two *E faecalis* strains, compared with 21.8 weeks for the five *E faecium* strains.

In addition, although both *E faecalis* strains were resuscitated after 24 hours in BHI broth, only two of the five *E faecium* strains showed apparent resuscitation, and then only after 72 hours.

The vancomycin resistance determinants of the VRE isolates after maximum time of recovery were shown to be stable by multiplex PCR.

After the maximum time of recovery, all strains also showed stability of DNA fingerprinting patterns by repetitive PCR using the ERIC1 primer.

Fitness cost

Table 2 shows a summary of the mean results of maximum growth rate and lag phase duration for VRE and VSE.

The difference between the mean maximum growth rates was not significant using the independent samples *t* test, either for all VRE versus all VSE ($p = 0.873$) or for vanB *E faecium* versus VSE *E faecium* ($p = 0.181$). In addition, there were no significant differences between mean values of lag phase duration ($p = 0.231$ and $p = 0.724$, respectively).

DISCUSSION

Although limited by the small numbers of strains tested, the experiment on survivability consistently indicated that VRE can survive for several weeks on dry cotton swabs at room temperature. The time of maximum recovery of *E faecium* strains was particularly prolonged. This differs from the results obtained by Wendt *et al* and Neeley and Maley, who found similar survival times for *E faecalis* and *E faecium* on various dry surfaces.[1 2]

Using a standard type of resuscitation procedure for VBNC, only two of five *E faecium* strains could be resuscitated, and then only with difficulty, whereas both *E faecalis* strains were easily resuscitated. Given the nature of the experiment and the lack of the application of strict criteria for the presence of VBNC, it is probable that the ''resuscitated'' strains of *E faecium* were actually revived injured survivors. In fact, the results probably illustrate the findings of Lleo *et al* that *E faecalis*, but not *E faecium*, can enter a VBNC state, whereas *E faecium* uses a starvation survival mechanism to enable at least some cells in the population to remain viable for prolonged periods.[7]

Unlike Johnsen *et al*, we found no fitness cost associated with vancomycin resistance.[11] This may be because the two studies had different designs: that by Johnsen *et al* involved competition between resistant and susceptible strains, whereas our present study involved measurement of lag phase duration and maximum growth rate in monoculture.

It may also be that the methods used in our study lack sensitivity. In most studies, fitness costs have been in the order of a few per cent and, in many cases, these were elicited

Table 2 Maximum growth rate and lag phase duration for VRE and VSE

	Maximum growth rate (ΔOD_{595}/min)	Lag phase duration (min)
All VRE	0.85 (0.12)	71.4 (16.8)
All VSE	0.86 (0.17)	81.1 (19.1)
vanB *E faecium*	0.85 (0.08)	77.5 (20.6)
VSE *E faecium*	0.98 (0.17)	80.9 (14.5)

Values are mean (SD).
OD_{595}, optical density at 595nm; VRE, vancomycin resistant enterococci; VSE, vancomycin sensitive enterococci.

Take home messages

- Vancomycin resistant enterococci (VRE) can survive for prolonged periods of time in a dry starvation state
- During this time they retain their genetic complement, including vancomycin resistance determinants, and show little or no fitness cost of vancomycin resistance
- The rate of entry required for VRE to become, and remain, endemic in the community is relatively small

in media deficient in carbon, phosphate, glucose, or other nutrients. It would be expected that differences would be less obvious in more complete media.

Another possible explanation is that the vanB mutation, unlike the vanA mutation investigated by Johnsen *et al*, does not incur a fitness cost.[11]

> ''It is probable that the 'resuscitated' strains of *Enterococcus faecium* were actually revived injured survivors''

It is also possible that the isolates had already undergone compensatory mutations, which had reduced or eliminated any fitness cost.

Although it is the accepted wisdom that antibiotic resistance genes and accessory elements engender a cost in the fitness of bacteria, the evidence for this being the case is, at best, modest. Resistance mutants found in bacteria from patients, as opposed to those artificially produced, have been found to have virtually no cost as measured by competition experiments, whether in vitro or in experimental animals.[12] Therefore, our results are not surprising.

The implication of the survivability study is that VRE can survive, by whatever mechanism, for considerable periods of time in a dry starvation state, while retaining their genetic complement, including genes for vancomycin resistance.

Furthermore, the fitness cost study indicates that it is unlikely that any fitness cost as a result of the acquisition of vancomycin resistance will significantly impair the ability of VRE to compete in the absence of the antibiotic; however, our study measured only growth kinetics, and other possible adverse effects of carriage of vancomycin resistance determinants—such as capacity for adherence to the human gut lining, resistance to immune attack, and production of virulence factors—were not investigated.

Assuming that any such adverse effects are not significant, the disappearance of VRE from the gastrointestinal tract of colonised individuals will probably occur over time, not because of a relative lack of fitness, but merely because of the relative abundance of susceptible strains compared with resistant strains. The higher the prevalence of resistant strains, the longer this will take to occur and, in fact, the greater the likelihood that it will not occur.

Taken together, the findings indicate that, given a sufficient (fairly small) rate of entry, VRE are likely to become, and remain, endemic in the general community.

Authors' affiliations
A A Ramadhan, E Hegedus, School of Biomedical Sciences, Faculty of Health Sciences, Cumberland Campus, PO Box 170, Lidcombe, NSW 1825, Australia

REFERENCES

1 **Wendt C**, Wiesenthal B, Dietz E, *et al*. Survival of vancomycin-resistant and vancomycin-susceptible enterococci on dry surfaces. *J Clin Microbiol* 1998;**36**:3734–6.
2 **Neeley AN**, Maley MP. Survival of enterococci and staphylococci on hospital fabrics and plastic. *J Clin Microbiol* 2000;**38**:724–6.
3 **Lleo MM**, Tafi MC, Canepari P. Nonculturable Enterococcus faecalis cells are metabolically active and capable of resuming active growth. *Syst Appl Microbiol* 1998;**221**:333–9.
4 **Lleo MM**, Tafi MC, Signoretto C, *et al*. Competitive polymerase chain reaction for quantification of nonculturable Enterococcus faecalis cells in lake water. *FEMS Microbiol Ecol* 1999;**30**:345–53.
5 **Lleo MM**, Perobon S, Tafi MC, *et al*. mRNA detection by reverse transcription-PCR for monitoring viability over time in an Enterococcus faecalis viable but nonculturable population maintained in a laboratory microcosm. *Appl Environ Microbiol* 2000;**66**:4564–7.
6 **Signoretto C**, Lleo MM, Tafi MC, *et al*. Cell wall chemical composition of Enterococcus faecalis in the viable but nonculturable state. *Appl Environ Microbiol* 2000;**66**:1953–9.
7 **Lleo MM**, Bonato B, Tafi MC, *et al*. Resuscitation rate in different enterococcal species in the viable but non-culturable state. *J Appl Microbiol* 2001;**91**:1095–102.
8 **Heim S**, Lleo MM, Bonato B, *et al*. The viable but nonculturable state and starvation are different stress responses of Enterococcus faecalis, as determined by proteome analysis. *J Bacteriol* 2002;**184**:6739–45.
9 **Lleo MM**, Bonato B, Signoretto C, *et al*. Vancomycin resistance is maintained in enterococci in the viable but nonculturable state and after division is resumed. *Antimicrob Agents Chemother* 2003;**47**:1154–6.
10 **Lenski RE**. Bacterial evolution and the cost of antibiotic resistance. *Int Microbiol* 1998;**1**:265–70.
11 **Johnsen PJ**, Simonsen GS, Olvisk O, *et al*. Stability, persistence, and evolution of plasmid-encoded VanA glycopeptide resistance in enterococci in the absence of antibiotic selection in vitro and in gnotobiotic mice. *Microb Drug Resist* 2002;**8**:161–70.
12 **Andersson DI**, Levin BR. The biological cost of antibiotic resistance. *Curr Opin Microbiol* 1999;**2**:489–93.