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CENTER

February 22, 2005

Division of Dockets Management (HFA-305)  
Food and Drug Administration  
5630 Fishers Lane, Room 1061  
Rockville, MD 20852

Re: Docket No. 2004N-0479  
Draft Risk Assessment of Streptogramin Resistance in *Enterococcus faecium*

We would like to take this opportunity to publicly comment on the FDA risk assessment regarding use of virginiamycin in food animals and the potential impact on streptogramin-resistant *E. faecium* infections in humans. We commend the FDA for attempting to quantify the human risks associated with virginiamycin use. However, we are concerned that one of the assumptions in the risk assessment model may underestimate the true risk for humans. In particular, we believe the FDA estimate of 10% may underestimate the actual proportion of streptogramin resistant *E. faecium* (SREF) that originates in the food supply (section 6.3.4).

Since mid-2002 we have been conducting an epidemiologic study to assess transfer of streptogramin resistance (and resistance determinants) through the food supply. This research has been funded by the Centers for Disease Control and Prevention. The study design included a cross-sectional survey of people admitted to community hospitals in four Midwestern cities, with collection of rectal swabs for detection of *E. faecium*. All enrollments were completed within 36 hours after hospital admission to ensure that fecal flora represented community carriage rather than nosocomial organisms. Each participant completed a detailed interview regarding diet and poultry exposures. Representative *E. faecium* isolates were assessed for susceptibility to quinupristin-dalfopristin (QD) by E test, and genetic factors associated with streptogramin resistance (e.g., vatD, vatE, ermB) were identified using PCR and sequencing. The same evaluations were performed on stool samples provided by a group of vegetarians who had not consumed poultry for at least one year.

To assess streptogramin-resistant *E. faecium* in the food supply, we also sampled retail poultry from typical grocery stores and alternative retail outlets (such as food co-ops and natural/organic food stores) where many of the poultry products were labeled as nonantibiotic-fed. Carcass washes were performed for isolation of *E. faecium*. Table 1 shows results of initial QD susceptibility testing for representative *E. faecium* isolates found in humans and poultry products.

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Table 1. Quinupristin-dalfopristin (QD) susceptibility and genetic markers in *Enterococcus faecium* isolated from different ecologic sources. Each observation pertains to a single person or poultry carcass colonized with *E. faecium*. NCCLS breakpoints were used to define QD susceptibility.

Group	Initial QD Susceptibility (E test)			Resistance Genes	
	Sensitive No. (%)	Intermediate No. (%)	Resistant No. (%)	vatE No. (%)	ermB No. (%)
Hospital recruits (n=105)	25 (24)	80 (76)	0	40 (38)	9 (9)
Vegetarians (n=65)	8 (12)	57 (88)	0	0	0
Typical retail poultry (n=77)	11 (14)	23 (30)	43 (56)	36 (47)	31 (40)
Alternative retail poultry (n=23)	11 (48)	9 (39)	3 (13)	3 (13)	2 (9)

The difference in the prevalence of vatE is striking when the hospital recruits (with high levels of poultry exposure) are compared with the vegetarians. However, neither group carried *E. faecium* with streptogramin resistance by E test. We performed additional tests to determine if QD resistance could be induced by pre-exposing isolates to subtherapeutic levels of virginiamycin (0.25 µg/mL for 24 hours). As described in the attached abstract and poster (presented at ICAAC 2004), pre-exposure to virginiamycin led to QD resistance in a substantial proportion of human isolates, and the inducible streptogramin resistance was strongly associated with the presence of vatE ( $p < .001$ ). None of the *E. faecium* isolates from vegetarians could be induced to the resistant phenotype.

For the analysis of epidemiologic risk factors in humans, we focused on two outcomes of interest: 1) presence or absence of vatE, and 2) presence or absence of QD resistant *E. faecium* (MIC  $\geq 4$  µg/mL) after low-dose virginiamycin pre-exposure (inducible resistance). Results were stratified by the presence or absence of antibiotic use in the month before enrollment, since recent antibiotic use was found to modify the effect of poultry exposures. In particular, poultry exposures were associated with streptogramin resistance determinants only in people who did not use antibiotics in the past month.

For people without recent antibiotic exposure, touching raw poultry and higher levels of poultry consumption (above the median) were associated with a higher prevalence ratios for vatE-containing *E. faecium* and inducible streptogramin resistance, although the results were not statistically significant when the analysis was limited to hospital recruits. The association was much stronger when the analysis was performed using both hospital recruits and vegetarians who were colonized with *E. faecium*. The prevalence of vatE-containing *E. faecium* was 29.2% in persons who had touched raw poultry in the past month, and 6.0% in those who had not (prevalence ratio 4.84, 95% confidence interval [CI] 1.69, 13.9). The prevalence of vatE-containing *E. faecium* was 40.0% in persons with a high level of poultry consumption (above the median for hospital recruits) and 4.5% in those with a low level of poultry consumption (prevalence ratio 8.9, 95% CI 2.97, 26.7).

Our results suggest that both touching raw poultry and poultry consumption are risk factors for carriage of *E. faecium* containing vatE. As shown in the table, we also found that vatE was common in *E. faecium* isolated from typical retail poultry, supporting the potential for foodborne transmission of this resistance determinant. This is also consistent with the complete absence of vatE in *E. faecium* isolates from vegetarians, and the low prevalence in alternative retail poultry with reduced exposure to antibiotic growth promoters.

Our data suggest that streptogramin resistance determinants can be acquired through the food supply. Our study was not designed to determine if the risk of a clinical SREF infection is increased by the presence of vatE-containing *E. faecium* in the gastrointestinal tract. As noted in the FDA risk assessment, there are other

unknown mechanisms of streptogramin resistance, and their potential for transmission through the food supply has also not been defined. However, we are concerned about the potential risk, particularly if QD use increases in hospitals, leading to greater selection pressure for streptogramin resistance.

In the proposed FDA risk assessment model, a 10% probability of origination in food pathways was selected based on limited data published by Willems et al (J Infect Dis 2000; 182:816-23). Our findings provide additional information to support a higher estimate. The proposed model uses a triangular distribution between 0% and 20%, peaking at 10%. A more conservative approach would be to use a distribution between 0% and 50%, peaking at 25%.

We are currently completing final multivariable analyses for this study, and results will be submitted for presentation and publication in the near future. In the meantime, we would be happy to discuss our methods and results with FDA staff in more detail.

Sincerely,



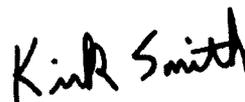
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# Quinupristin-Dalfopristin Resistance in Human *Enterococcus faecium* Increases with Pre-Exposure to Virginiamycin

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## REVISED ABSTRACT

**Background:** Virginiamycin (Vm) is used as a growth promoter in poultry. It selects for cross-resistance to another streptogramin, quinupristin-dalfopristin (QD), which is effective against vancomycin-resistant *E. faecium* (EF) QD-resistant EF (QRE) are commonly found on poultry farms and retail poultry products but are rarely isolated from humans. We conjectured that Vm may induce QD resistance, and the observed low prevalence of QDRE in humans may reflect the lack of recent Vm exposure.

**Methods:** Rectal swabs obtained from patients at the time of hospital admission were evaluated for *E. faecium* biochemistries and a species-specific PCR. QD susceptibility was measured by E-test and by a pre-exposure assay. In this assay  $1 \times 10^6$  EF were cultured in a pre-exposure BHI broth containing 0.25 µg/ml Vm at 37°C for 24 hr.  $1 \times 10^6$  EF were then transferred to a challenge BHI broth containing 8 µg/ml QD. An isolate was classified as resistant if its final density in stationary phase at 24 hr in the challenge broth was >10% of its growth in a no Vm pre-exposure no-QD control broth.

**Results:** Of 116 EF isolates from 116 patients 30 were QD-susceptible, 36 had intermediate resistance and 0 were resistant by E-test (i.e. all had MICs < 4 µg/ml). In contrast, when these same isolates were pre-exposed to Vm and challenged with 8 µg/ml QD, 67 isolates had growth < 10% of their no-QD control and 49 isolates had > 10% growth. 14 isolates had ≥ 30% growth. Controls with the QD challenge but without Vm pre-exposure did not grow, indicating that Vm pre-exposure was necessary for the resistant phenotype. Three isolates became QD resistant after only 3 hr Vm pre-exposure. PFGE confirmed that the increase in QD resistance after Vm pre-exposure was not due to a contaminating isolate or selection for a hidden genotype.

**Conclusion:** Pre-exposure to Vm increased the QD resistance of nearly half of human EF isolates suggesting that Vm induces QD resistance.

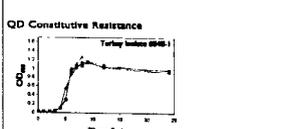
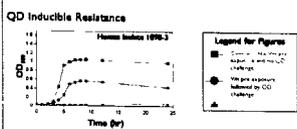
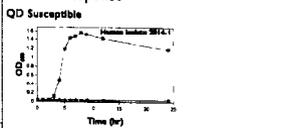
## INTRODUCTION

**Observations Leading to Study Hypothesis**  
The present study is a component of a larger epidemiologic investigation on risk factors for human carriage of streptogramin resistant *E. faecium*. This latter study included quinupristin-dalfopristin (QD) susceptibility testing of *E. faecium* isolates from people and retail poultry products in four Midwestern communities and a turkey farm near one of these communities. As commonly practiced in the poultry industry, the turkey farm used virginiamycin (Vm) a streptogramin compound related to QD as a growth promoter. All 37 turkey isolates were QD resistant and among 77 retail poultry isolates 41 (56%) were QD resistant. Remarkably, among 116 isolates from 116 people there were QD resistant even though these people likely purchased and consumed poultry products carrying QD resistant *E. faecium*. These observations led to the hypothesis that exposure of QD resistance is diminished with increasing time since exposure to Vm in the poultry farm environment and is inversely to Vm would revert some human *E. faecium* isolates to the QD resistant phenotype.

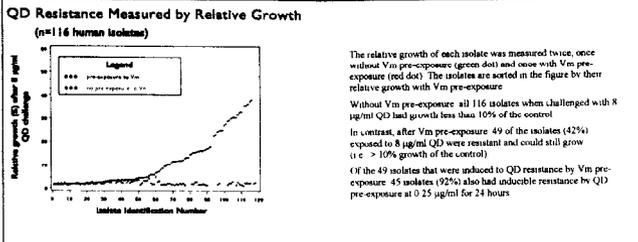
## METHODS

Rectal swabs were obtained from patients within 36 hours of admission into four Midwestern hospitals during 2002-2003. *E. faecium* was isolated on CNA agar and identified by standard biochemistries followed by PCR for a species-specific *efl* amplicon (Dutka-Malen et al 1995). Presence of the acetyltransferase gene *vatE* was assessed by PCR following the method of Warner and Witte (1999). Vm pre-exposure was accomplished by culturing  $1 \times 10^6$  bacteria/ml in 5 ml BHI broth containing 0.25 µg/ml Vm at 37°C for 24 hours. QD susceptibility was measured by three methods: 1) Minimum inhibitory concentration (MIC) by E-test; 2) Minimum bactericidal concentration (MBC) by the time-kill method (NCCLS 1999) using QD concentrations of 4, 8, 16 and 32 µg/ml; 3) Relative growth, in which QD susceptibility was expressed as the ratio of the isolate's final density after 24 hours in a challenge BHI broth containing 8 µg/ml QD, over the final density of the same isolate after 24 hours in BHI broth without QD. Bacterial density was measured as optical density (600 nm).

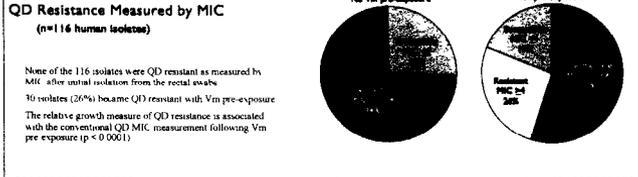
## Interpretation of the Relative Growth Response



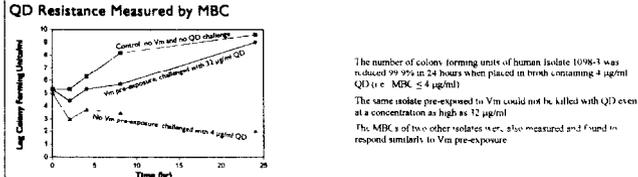
## EFFECT OF VIRGINIAMYCIN PRE-EXPOSURE ON QD RESISTANCE



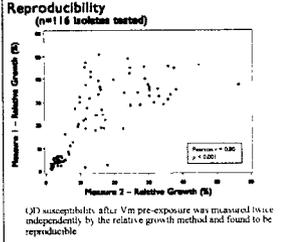
## QD Resistance Measured by MIC (n=116 human isolates)



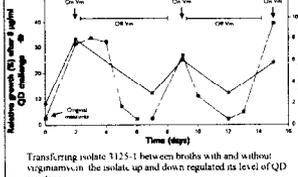
## QD Resistance Measured by MBC



## RESISTANCE INDUCIBILITY OR EXPERIMENTAL ARTIFACT?



## Up and Down Regulating Resistance



Transferring isolate 3125-1 between broths with and without virginiamycin, the isolate up and down regulated its level of QD resistance. Vm exposure for 24 hours increased QD resistance when Vm was removed QD resistance diminished after 3 days (i.e. 3 transfers). Two other isolates were evaluated and responded similarly.

**Contamination and Selection**  
The pulsed-field gel electrophoresis patterns of the 50 induced isolates were distinct from each other and at the end of the relative growth assays were indistinguishable from their no Vm, no QD controls, confirming that the observed resistance was not from a contaminating resistant isolate, or selection for a hidden resistant genotype.

**Point Mutations**  
Genetic point mutations are an unlikely explanation for the change in QD resistance because: 1) The initial inoculum of bacteria ( $1 \times 10^6$ ) was low relative to bacterial mutation rates; 2) Inoculation in Vm was no longer than 24 hours, minimizing the chance for mutations.

## ASSOCIATION OF *vatE* WITH INDUCIBLE QD RESISTANCE

Vm Pre-exposure	resE	n	Median Relative Growth (%) after QD Challenge	p-value Wilcoxon Test
No	Present	46	2.0	0.63
	Absent	70	2.0	
Vm	Present	46	16.0	<0.001
	Absent	70	3.5	

The acetyltransferase gene *vatE* was strongly associated with QD resistance measured by relative growth, only if the isolates were pre-exposed to Vm. Similarly when measured by MIC, *vatE* was associated with QD resistance only after the isolates were pre-exposed to Vm (chi-square,  $n = 116$ ,  $p$ -value = 0.005).

## CONCLUSIONS

Exposure to a low concentration of Vm (0.25 µg/ml) for 24 hours substantially increased the level of QD resistance in nearly half of the human *E. faecium* isolates. QD resistance following Vm pre-exposure was associated with the presence of the resistance element *vatE*, although some resistant isolates lacked *vatE*. The QD resistance response of 116 isolates to Vm pre-exposure was reproducible, the QD resistance level could be raised or lowered by adding or removing Vm from the cultures and there was no evidence of contamination by or selection for resistant genotypes, all of which suggests that the mechanism of QD resistance was inducible.

## ACKNOWLEDGEMENT AND DISCLAIMER

The authors would like to acknowledge the contributions of Joanne Barakat, Jeff Bender, Phil Bertz, Dave Borsari, Meghan C. Byrne, Selma Jaman, Krystal Peterson, Christal Lynn Iverson, Jane Salschell, Kirk Smith, the Epidemiology Research Center Study Unit, and the Biostatistics and Biominformatics Core Data Unit. This research was supported by CDC Cooperative Agreement R31CE000634. Its contents are solely the responsibility of its authors and do not necessarily represent the official views of CDC.

## REFERENCES

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National Committee for Clinical Laboratory Standards. 1999. Methods for determining bactericidal activity of antimicrobial agents. Document M26-A. National Committee for Clinical Laboratory Standards, Wayne, PA.  
Warner, G. and W. Witte. 1999. Characterization of a new enterococcal gene *vatE* encoding a putative acetyltransferase conferring resistance to streptogramin compounds. *Antimicrob. Agents Chemother.* 43:1813-1814.

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## REVISED ABSTRACT

**Background:** Virginiamycin (Vm) is used as a growth promoter in poultry. It selects for cross-resistance to another pleiotropic quinupristin-dalfopristin (QD), which is effective against common cross-resistant *E. faecium* (QD-resistant *E. faecium* (QDRE)) are commonly found on poultry farms and retail poultry products but are rarely isolated from humans. We conjectured that Vm may induce QD resistance and the observed low prevalence of QDRE in humans may reflect the lack of recent Vm exposure.

**Methods:** Rectal swabs obtained from patients at the time of hospital admission were evaluated for *E. faecium* and a species-specific PCR QD susceptibility was measured by E-test and by a pre-exposure assay. In this assay,  $1 \times 10^6$  CFU were cultured in a pre-exposure BHI broth containing 0.25 µg/ml Vm for 24 hr.  $1 \times 10^6$  CFU were then transferred to a challenge BHI broth containing 8 µg/ml QD. An isolate was classified as resistant if its final density in stationary phase at 24 hr in the challenge broth was >10% of its growth in a no Vm pre-exposure, no-QD control broth.

**Results:** Of 116 *E. faecium* isolates from 116 patients, 30 were QD-susceptible, 86 had intermediate resistance, and 0 were resistant by E-test (i.e. all had MICs < 4 µg/ml). In contrast, when these same isolates were pre-exposed to Vm and challenged with 8 µg/ml QD, 67 isolates had growth < 10% of their no-QD control and 49 isolates had > 10% growth, 14 isolates had > 30% growth. Controls with the QD challenge but without Vm pre-exposure did not grow, indicating that Vm pre-exposure was necessary for the resistant phenotype. Three isolates became QD resistant after only 3 hr Vm pre-exposure. PFGE confirmed that the increase in QD resistance after Vm pre-exposure was not due to a contaminating isolate or selection for a hidden genotype.

**Conclusion:** Pre-exposure to Vm increased the QD resistance of nearly half of human *E. faecium* isolates, suggesting that Vm induces QD resistance.

## INTRODUCTION

**Observations Leading to Study Hypothesis**  
The present study is a component of a larger epidemiologic investigation on risk factors for human carriage of streptogramin resistant *E. faecium*. This study included quinupristin-dalfopristin (QD) susceptibility testing of *E. faecium* isolates from people and retail poultry products in four Midwestern communities and a turkey farm near one of these communities. As commonly practiced in the poultry industry, the turkey farm used virginiamycin (Vm), a streptogramin compound related to QD, as a growth promoter. All 47 turkey isolates were QD resistant and among 77 retail poultry isolates 43 (56%) were QD resistant. Remarkably, among 116 isolates from 116 people none were QD resistant even though these people likely purchased and consumed poultry products carrying QD resistant *E. faecium*. These observations led to the hypothesis that exposure of QD resistance diminished with increasing time since exposure to Vm in the poultry farm environment and re-exposure to Vm would select some human *E. faecium* isolates to the QD resistant phenotype.

## METHODS

Rectal swabs were obtained from patients within 36 hours of admission into four Midwestern hospitals during 2002-2003.

*E. faecium* was isolated on CNA agar and identified by standard biochemical tests followed by PCR for a species-specific *ddl* amplicon (Datta-Malen et al. 1995).

Presence of the *actA* transference gene *vatE* was assessed by PCR following the method of Werner and Witte (1999).

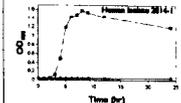
Vm pre-exposure was accomplished by culturing  $1 \times 10^6$  bacteria/ml in 5 ml BHI broth containing 0.25 µg/ml Vm at 37°C for 24 hours.

QD susceptibility was measured by three methods:

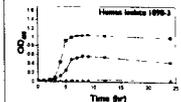
- 1) Minimum inhibitory concentration (MIC) by E-test
- 2) Minimum bactericidal concentration (MBC) by the time-kill method (NCCLS 1999) using QD concentrations of 4, 8, 16 and 32 µg/ml
- 3) Relative growth in which QD susceptibility was expressed as the ratio of the isolate's final density after 24 hours in a challenge BHI broth containing 8 µg/ml QD over the final density of the same isolate after 24 hours in BHI broth without QD. Bacterial density was measured as optical density 600 nm.

## Interpretation of the Relative Growth Response

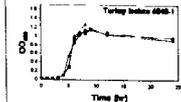
### QD Susceptible



### QD Inducible Resistance

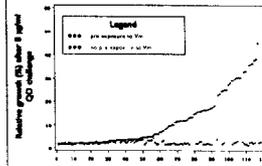


### QD Constitutive Resistance



## EFFECT OF VIRGINIAMYCIN PRE-EXPOSURE ON QD RESISTANCE

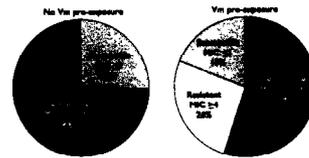
### QD Resistance Measured by Relative Growth (n=116 human isolates)



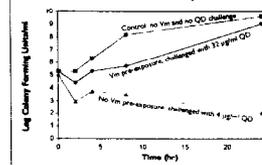
The relative growth of each isolate was measured twice, once without Vm pre-exposure (green dot) and once with Vm pre-exposure (red dot). The isolates are sorted in the figure by their relative growth with Vm pre-exposure. Without Vm pre-exposure, all 116 isolates when challenged with 8 µg/ml QD had growth less than 10% of the control. In contrast, after Vm pre-exposure, 49 of the isolates (42%) exposed to 8 µg/ml QD were resistant and could still grow (i.e. > 10% growth of the control). Of the 49 isolates that were induced to QD resistance by Vm pre-exposure, 45 isolates (92%) also had inducible resistance by QD pre-exposure at 0.25 µg/ml for 24 hours.

### QD Resistance Measured by MIC (n=116 human isolates)

None of the 116 isolates were QD resistant as measured by MIC after initial isolation from the rectal swabs. 30 isolates (26%) became QD resistant with Vm pre-exposure. The relative growth measure of QD resistance is associated with the conventional QD MIC measurement following Vm pre-exposure (p < 0.0001).



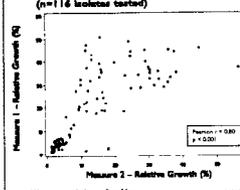
### QD Resistance Measured by MBC



The number of colony forming units of human isolate 1098-3 was reduced 99.9% in 24 hours when placed in broth containing 4 µg/ml QD (i.e. MBC = 4 µg/ml). The same isolate pre-exposed to Vm could not be killed with QD even at a concentration as high as 32 µg/ml. The MICs of two other isolates were also measured and found to respond similarly to Vm pre-exposure.

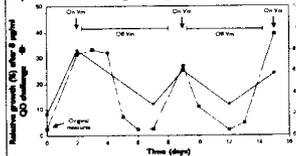
## RESISTANCE INDUCIBILITY OR EXPERIMENTAL ARTIFACT?

### Reproducibility (n=116 isolates tested)



QD susceptibility after Vm pre-exposure was measured twice independently by the relative growth method and found to be reproducible.

### Up and Down Regulating Resistance



Transferring isolate 3125-1 between broths with and without virginiamycin, the isolate up and down regulated its level of QD resistance.

Vm exposure for 24 hours increased QD resistance when Vm was removed QD resistance diminished after 3 days (i.e. 3 transfers). Two other isolates were evaluated and responded similarly.

### Contamination and Selection

The pulsed-field gel electrophoresis patterns of the 50 induced isolates were distinct from each other and at the end of the relative growth assay were indistinguishable from their no Vm, no QD controls, confirming that the observed resistance was not from a contaminating resistant isolate or selection for a hidden resistant genotype.

### Point Mutations

Genetic point mutations are an unlikely explanation for the change in QD resistance because: 1) The initial inoculum of bacteria ( $1 \times 10^6$ ) was low relative to bacterial mutation rates. 2) Incubation in Vm was no longer than 24 hours minimizing the chance for mutations.

## ASSOCIATION OF *vatE* WITH INDUCIBLE QD RESISTANCE

Vm Pre-exposure	MIC	n	Relative Growth (%) after QD Challenge	p-value Wilcoxon Test
No	Present	46	2.0	0.63
	Absent	70	2.0	
Yes	Present	46	15.0	<0.001
	Absent	70	1.5	

The *actA* transference gene *vatE* was strongly associated with QD resistance measured by relative growth only if the isolates were pre-exposed to Vm. Similarly, when measured by MIC, *vatE* was associated with QD resistance only after the isolates were pre-exposed to Vm (chi-square n = 116, p-value = 0.005).

## CONCLUSIONS

Exposure to a low concentration of Vm (0.25 µg/ml) for 24 hours substantially increased the level of QD resistance in nearly half of the human *E. faecium* isolates.

QD resistance following Vm pre-exposure was associated with the presence of the resistance element *vatE*.

The QD resistance response of 116 isolates to Vm pre-exposure was reproducible; the QD resistance level could be raised or lowered by adding or removing Vm from the cultures and there was no evidence of contamination by or selection for resistant genotypes, all of which suggests that the mechanism of QD resistance was inducible.

## ACKNOWLEDGEMENT AND DISCLAIMER

The authors would like to acknowledge the contributions of Joanne Barfknecht, Jeff Bender, Paul Benz, Dave Borral, Meghan Cleverley, Selma Jaman, Kristin Pederson Chalmers, Lynn Viscusi, Jon Scherfeld, Kirk Smith, the Epidemiology Research Center Study Unit, and the Biorobotics and Biomaterials Core Data Unit. This research was supported by CDC Cooperative Agreement RS1ACR520634. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of CDC.

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Werner, G., and W. Witte. 1999. Characterization of a new *mevA* gene of *Streptococcus pneumoniae* conferring resistance to quinupristin-dalfopristin. *Antimicrob. Agents Chemother.* 43:1811-1814.