EVALUATION OF THE SURVIVAL OF VANCOMYCIN-RESISTANT ENTEROCOCCI (VRE) ISOLATED FROM CHICKENS AND POSSIBLE INACTIVATION BY IN-USE CONCENTRATION OF LINDORES-30®, ECOS TIMSEN® AND OMNICIDE®

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ABSTRACT. Vancomycin-resistant enterococci (VRE) are well-known ascendant nosocomial pathogens. The recent detection of epidemiologic strain carrying *vanA* gene in the community of people working with animals and in chickens has brought to the forefront the potential public health danger posed by these organism. The farm environment is a major source of VRE persistence in poultry farms. We carried out survival test to test the survival of the VRE isolates on dry condition and surface test to evaluate the inactivation of the isolates by in-use concentration of commonly used disinfectants. In the survival test, all isolates survived for at least 4 weeks in colony counts of ($1.00 \times 10^3 - 3.86 \times 10^3$ CFU/ml) under clean condition and ($1.00 \times 10^3 - 2.02 \times 10^4$) for soiled condition. Those that were suspended in 5% BSA solution to mimic organic matter load as obtainable on farms survived for at least 8 weeks at ($1.54 \times 10^2 - 1.34 \times 10^3$ CFU/ml). In the surface test, inactivation of VRE isolates by in-use concentration of Lindores®, Omnicide® and Ecos Timsen® was tested using the European surface test (EST). All the tested disinfectants were active against the VRE isolates on both the standard test surface (stainless steel) and our test surface (wooden). The results shows microbiocidal effects (ME) for test disinfectants, i.e. the $\log_{10}$ CFU of micro-organisms compared between test biocide and control treated with distilled water, after 7 min of exposure as follows; Lindores® active on both surfaces 5.24 and 3.17, Ecos Timsen® active significantly on steel 4.90 than wood 2.98 and Omnicide® significantly less active on stainless steel 2.40 than on wood 3.50.

Keywords: VRE, disinfectants, chickens, microbiocidal effect, survival, European surface test.

INTRODUCTION

Ever since the discovery of the first case of vancomycin-resistant Enterococcus (VRE) infection in Britain in 1986 (Uttley *et al.*, 1988), the organism has grown in reputation and importance as an ascendant nosocomial pathogen. Livestock and livestock related

products has been asserted as one of the modes by which humans can get infected with VRE (Bates, 1997), which then led to the importation restrictions on VRE contaminated products from Malaysia into the neighbouring country (Zaini *et al.*, 2000a; Zaini *et al.*, 2000b). In Malaysia, the presence of VRE possessing the clinically relevant *vanA* and *vanB* genes in chickens, pigs and community of people working with animals had been confirmed recently (Getachew *et al.*, 2008; Getachew *et al.*, 2009; Getachew *et al.*, 2010). Although the direct link and association between the VRE in animals to that of humans remains controversial, the presence of VRE in farms is a concern among livestock poultry farmers. Environmental contamination has been implicated as one of the major source of infection in many hospitals outbreak (Lemmen *et al.,* 2004; Martinez *et al.*, 2003) and the farm environment has been identified as the major reservoir of VRE persistence in the poultry farm (Garcia-Migura *et al.*, 2007). In this study, we investigate the survival of VRE isolates from chickens in an experimental setting which mimics the hot and humid tropical environment.and evaluate their inactivation by the in-use concentration of commonly used disinfectants.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Four VRE isolates (2 *Enterococcus faecium*, 2 *Enterococcus faecalis*) from healthy chickens and two reference isolates (ATCC reference strains), all resistant to vancomycin were used for this experiment (Table 1). These isolates have been biochemically and molecularly characterized in previous work (Getachew, 2010). The isolates were stored at -20°C in Brain Heart Infusion (BHI) containing 20% glycerol broth. All the isolates are grown on BHI agar at 37° ± 2°C for 24h.

Test inocula

The isolates were washed and suspended in sterile distilled water or in 5% BSA to create the 'clean' and 'soiled' conditions,

Table 1. Vancomycin-resistant enterococcus isolates used in the study, their origin and vancomycin resistance level.

Isolate	Source	MIC (µg/ml) Vancomycin	origin
Enterococci faecium PY60	Survey	64	Chicken
Enterococci faecium PY46	Survey	124	Chicken
Enterococci faecalis PY135	Survey	256	Chicken
Enterococci faecalis PY07	Survey	64	Chicken
Enterococci faecium ATCC51559	ATCC	>256	ATCC
Enterococci faecalis ATCC51299	ATCC	24	ATCC

respectively. The final concentration of the inocula was determined by dilution series/turbidimetry and using McFarland standard to averaged $3x10^8$ CFU/ml

Survival Test

The methodology for this study was adopted from Wendt *et al*., (1998) with slight modification.

Surface inoculation and culturing:

We employed wooden surface because this type of surface is prevalent in the farm environments. About 540 25 cm^2 wooden chips were autoclaved and then contaminated with 0.1 ml of the bacterial suspension. All samples were stored at 32°C ± 2°C with 80% ± 10% relative humidity in a dust protected chamber. At various time intervals – time 0 (immediately after drying), at 4 hours, 1 day and 1, 2, 4, 8, 12 and 16 weeks, viable cells were recovered from the contaminated surfaces. Five wooden chips samples per isolate were selected at random at each time interval; then placed in 250 ml beaker containing 100 ml of 0.9% NaCl solution and 5 mm diameter glass beads with the inoculated surface in contact with the glass beads. They were then shaken for 5 min in a shaking hot water bath (200 strokes min^{-1} stroke amplitude of 7.5 cm) and the number of viable isolates in the recovery media was determined using serial dilution and membrane filtration technique as described by Slanetz and Bartley (1957).

Data analysis

All data collected were recorded and analysed using Microsoft Excel. The number of viable bacteria on per surface was calculated using the formula below:

$$X = N/D$$

Where

X, the presumed number of viable cells counted

N, number of viable cells counted and

D, the dilution volume

Alternatively,

$$X = N \times DF$$

Where

DF, the dilution factor which is the reciprocal of D

Surface Test

This was performed according to the harmonised European surface test (Bloomfield *et al*., 1993) and as performed by Block *et al*., (2000).

Surfaces

Two surfaces (stainless steel and wood) in two conditions (clean and soil) were used in this experiment; small circular stainless steel disc 2 cm in diameter and a 4 cm^2 wooden chip were used as standard test surfaces. The surfaces were sterilised before use.

Neutralising medium

Dey and Engleys neutralising media was used as a recovery media to neutralize the effect of the disinfectant.

Disinfectant solutions: three disinfectants(Ecos Timsen®, Lindores*-30® and Omnicide®) were used in their in-use concentration as recommended by the manufacturers.

Predetermined exposure time: Preliminary experiments were performed to select the optimum exposure time for the disinfectants that resulted in at least a 3 log reduction of the initial number of VRE used in the study (Block *et al.*, 2000). The test was carried out with 2 randomly selected isolates (*E. faecium* and *E. faecalis*). The isolates were tested against all 3 disinfectants; Ecos Timsen®, Lindores*-30® and Omnicide® using the method as described below. Three hundred and sixty stainless steel discs were prepared to allow assays at 1 minute interval for up to 10 min. An exposure time to achieve a microbiocidal effect (ME) > 3 for most

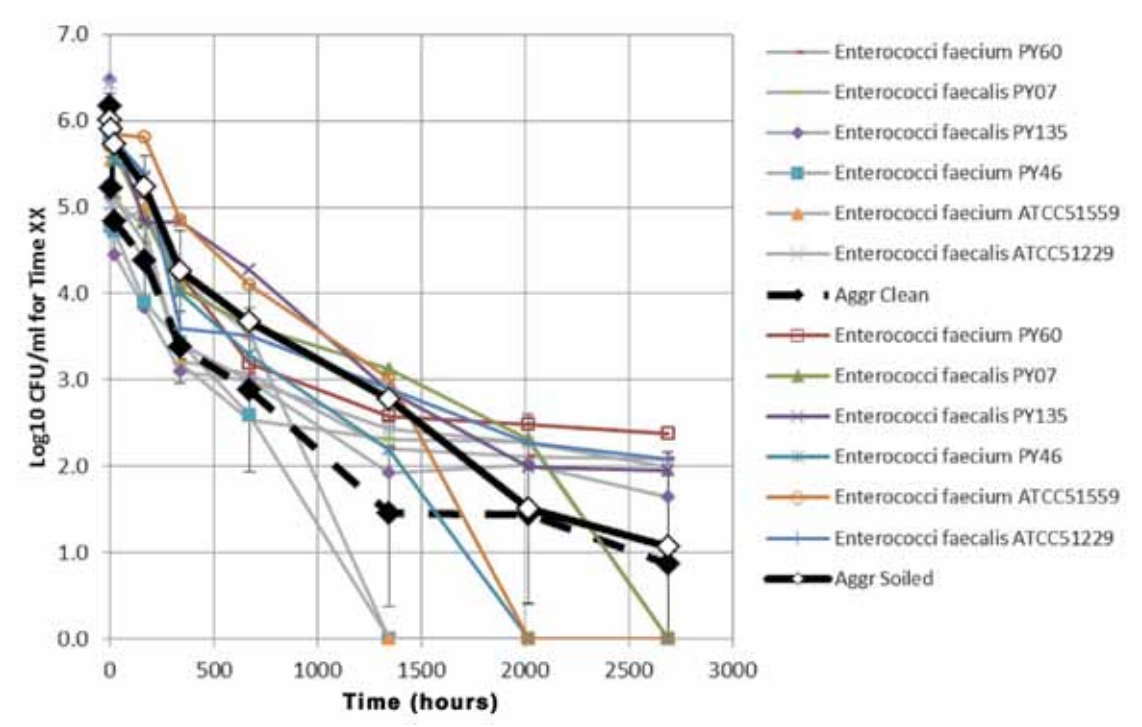

Figure 1. Survival of VRE over a 16-weeks sampling period when exposed to a typical tropical environmental condition of 30±2oC and 80±10% relative humidity in a dust protected chamber. Black bold lines represent aggregate counts for all species in different conditions; error bars represents ±1 Standard Deviation from the mean, coloured lines represents soiled condition and grey lines for clean condition.

isolates was chosen for each combination of isolate and disinfectant as shown below (Figure 2). The ME was calculated as indicated below (Anon. 2007).

Surface inoculation and culturing

The inocula and the biocide solution were equilibrated to 25°C before use. 100 µl of the inocula containing 1 – 3 × 10^8 CFU/ml was dropped on the surface and dried in a fan assisted incubator for 1 hour. Sample of disinfectant or distilled water was dropped on the surface to cover the test film. After a contact time of 7 min the sample was placed in a 50 ml-beaker containing 10 ml NM together with 3 mm Θ glass beads and placed in a reciprocal shaker for 20 minutes. Viable cells were recovered from the media using a pair of serial dilution and membrane filtration technique.

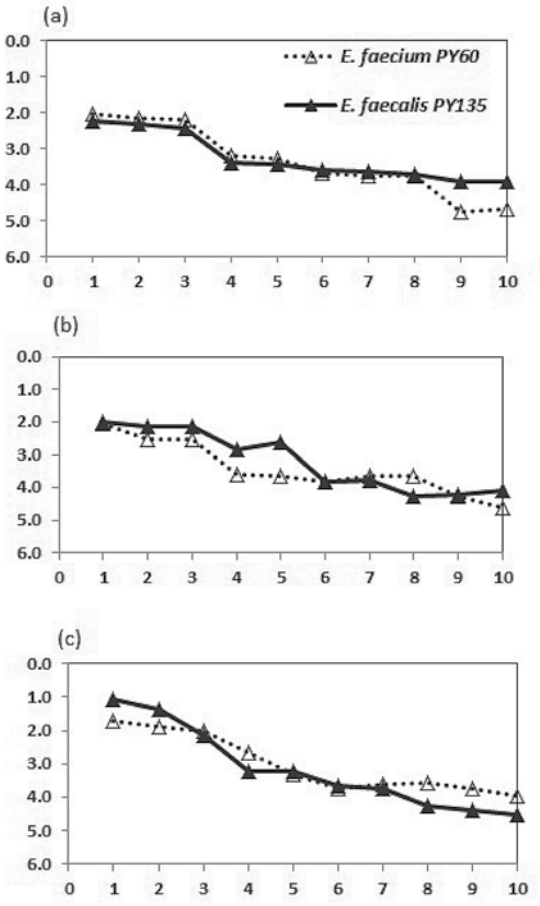

Figure 2. Killing kinetics of (a) Ecos Timsen (b) Lindores and (c) Omnicide on strain of vancomycin resistant *E. faecium* and *E. faecalis* each dried on stainless steel surface.

Data analysis

The results were expressed as ME which is the log reduction of bacterial count due to the action of a disinfectant. The ME is calculated as the $\log_{10}$ value of the counts after exposure to the disinfectant (Nd) subtracted from the $\log_{10}$ value of the counts after exposure to water as control (NC);

$$ME = \log_{10}(NC) - \log_{10}(Nd)$$

RESULTS

Survival Test

All isolates survived with colony counts of 1.00 × 10^3 – 5.06 × 10^3 CFU/ml for at least 4 weeks under soiled condition and of 1.00 × 10^3 – 3.86 × 10^3 CFU/ml for clean condition, those in soiled condition survived at 1.54 x 10^2 – 1.34 x 10^3 CFU/ml for at least 8 weeks. Three isolates, two isolated from chickens and one reference

Table 2. Mean Microbiocidal Effect (ME) for each biocide and test surface ± 1SD

Test Biocide	Test Surface	Mean ME±SD	Sig.	Average ME±SD
Ecos Timsen®	Stainless steel disc	4.90±1.61	0.00	4.08±1.55
	Wooden chip	2.98±0.46		
Lindores®	Stainless steel disc	5.24±1.21	0.00	4.36±1.45
	Wooden chip	3.17±0.81		
Omnicide®	Stainless steel disc	2.40±0.70	0.00	3.00±0.87
	Wooden chip	3.50±0.67		

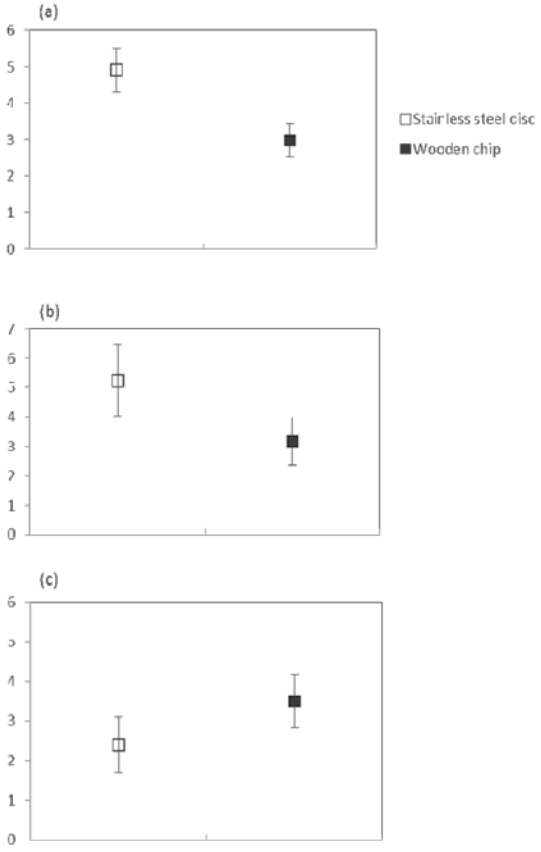

Figure 3. Mean microbiocidal effect (ME) determinations of (a) Ecos Timsen, (b) Lindores*-30 and (c) Omnicide for vancomycin resistant enterococci (□ mean ME± 95% confidence interval)

strain, survived for the whole duration of the sampling in both conditions. Using the $\log_{10}$ of the colony counts, 12 survival curves were plotted for the 6 isolates in two conditions (Figure 1). Those in the clean condition shows an aggregate of 5.3 $\log_{10}$ steps reduction in CFU/ml counts and those in soiled conditions showed a 4.9 $\log_{10}$ steps reduction at the end of the 16 weeks sampling period.

Surface Test

We found that that Lindores® > Ecos Timsen® > Omnicide® in efficacy against the isolates with average MEs of 4.36, 4.08 and 3.00 irrespective of the test surface used (Table 2). Ecos Timsen® shows an ME of 4.90 ± 1.61 on stainless steel surface and 2.98 ± 0.46 on wooden surface, Lindores® an ME of 5.24 ± 1.21 on stainless steel and 3.17 ± 0.81 on wooden surface whereas Omnicide® gave an ME of 2.40 ± 0.70 on stainless steel surface and 3.50 ± 0.67 on wooden surface (Figure 3).

DISCUSSIONS AND CONCLUSIONS

Very few experiments have been performed on VRE with regards to their survival in the environment. Therefore, we find it challenging to compare our findings to others. The few studies that are available may not be comparable because of the different strains, type of surface and conditions used in the study. However, in general our results are consistent with other studies that found VRE to survive for period between 5 days to 4 months on clinically relevant materials and surfaces (hospital fabrics, plastics, blood pressure cuffs, counter tops, drawsheet, enteral feed, bedrails, urine container, telephones, stethoscope, ceramic, poly vinyl chloride (PVC), stainless steel and rubber) at 22°C ± 2°C with 50% ± 5% relative humidity (Bonilla *et al*., 1996; Neely and Maley, 2000; Noskin *et al*., 1995; Wendt *et al*., 1997; Wendt *et al*., 1998), the huge range in survival time can be attributed to diverse origin of isolates, since isolates from dry sources such as pillows and countertops are known to survive longer than isolates from wet sources such as urine (Wendt *et al*., 1997). In our investigation, all isolates survived for at least 4 weeks in clean condition (suspended in distilled water) and for up to 8 weeks in soiled condition (suspended in 5% BSA) on the wooden surface at the temperature of 30°C ± 2°C and relative humidity of 80 ± 10% to mimic the Malaysian climate. The survival times of bacteria is known to increase with the increase in relative humidity (11 days at 31% RH and 4 days at 10%) for *Acinetobacter baumannii* and the presence or absence of protein (43 days when suspended in 7% BSA as against 3 days when suspended in distilled water) for *Enterococcus* spp. (Jawad *et al*., 1996). Out of the 6 isolates studied, 3 (*Enterococci faecium* PY60, *Enterococci faecalis* PY135 and *Enterococci faecalis* ATCC51229) survived the whole 4 months study period.

Disinfectants are rated to have high activity when they show MEs of >5.4 to >6.6 (indicating no detectable survivors), intermediate activity with MEs between 2 and 5 and low activity showing MEs of between 0.5–3.0 (Bloomfield *et al*., 1993). The result for the surface test shows that Ecos Timsen® and Lindores® possess intermediate activity against the isolates on stainless steel surface and low to intermediate activity on wooden surface, Omnicide® on the other hand shows low activity on stainless steel surface and intermediate activity on wooden surface. There was a significant difference in the ME values between the two test surfaces for each disinfectant ($p = 0.01$). Overall, the results show that the disinfectants were less effective against the isolates on wooden surface with ME <4. This could be attributed to the ability of the wooden surfaces to absorb the inoculum (Ak *et al*., 1994), thus making it difficult for the disinfectant to act effectively against the isolates.

In conclusion, environmental contamination in the farm presents a hazard for the persistence and endemicity

of VRE. VRE from chicken may survive for more than 4 months in the local climate and environment. Furthermore, the low to intermediate activity of the in-use concentration of the disinfectants will not completely remove VRE from the environment especially when dried on wooden surface. The presence of organic soiling such as that which exists in farm environments will further confound the activity of the disinfectant (Anon., 2007; Russell, 2008), making it more difficult to eliminate VRE from these environment.

The conditions set for the surface test for this study is without mechanical action (Anon., 2007), hence cleaning procedures with detergent involving mechanical actions such as mopping or scrubbing will greatly improve the activity of the disinfectants. We recommend that further tests should be perform in the presence of organic soiling to evaluate the disinfection procedure in real on-farm scenario.

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