

## VANCOMYCIN RESISTANCE OF *ENTEROCOCCUS* SPECIES ISOLATED FROM RAW MILK AND SOME CHEESES

BY

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### SUMMARY

Susceptibility of isolated enterococci against different vancomycin concentrations 10  $\mu\text{g}$ , 20  $\mu\text{g}$ , 30  $\mu\text{g}$  up to 80  $\mu\text{g}$  was studied. Only 18.75%, 3.75% and 3.75% of tested enterococci isolates were resistant to 10  $\mu\text{g}$ , 20  $\mu\text{g}$  and 30  $\mu\text{g}$ , vancomycin concentrations respectively, where moderate susceptibility with inhibition zones of  $>0.6$  to  $<1.5\text{cm}$  were noticed with 72.5%, 66.25% and 23.75% by previous vancomycin concentrations respectively. Finally, 8.75%, 30% and 72.5% of isolated *Enterococcus* species were sensitive to 10  $\mu\text{g}$ , 20  $\mu\text{g}$  and 30  $\mu\text{g}$ , respectively. All milk, Ras cheese and Karish cheese isolates were sensitive to vancomycin concentrations of 20  $\mu\text{g}$  and 30  $\mu\text{g}$  as compared with Domiati cheese, isolates where 3 strains, namely *E. faecalis* Dc2-2, *E. faecalis* Dc3-1 and *E. faecalis* Dc5-2 respectively were resistant. However, these strains exhibit less resistance towards high vancomycin concentration up to 80  $\mu\text{g}$ . Inhibition zones of 0.8 cm was observed with very low numbers of tested isolates which indicate the unpowerful effect of vancomycin concentrations of 10  $\mu\text{g}$  and 20  $\mu\text{g}$  actually 11 and 6 isolates respectively. It is clear from these results that 16 strains were the most sensitive (inhibition zone  $\geq 2$  cm), and 11 strains gave lower inhibition zone (1.2 or 1.3 cm) which could be considered as moderate sensitive to vancomycin.

### INTRODUCTION

Vancomycin was first used in clinical arena in 1972, while the first vancomycin – resistant enterococci (VRE) was recognized only 15 years later (Metan et al., 2005) and were first detected in the UK and France in 1986 (Leclercq et al., 1988 and Uttely et al., 1988).

The VRE is considered to be serious problem and an important cause of nosocomial infections over worldwide (Koluman et al., 2009). Over a 15 year period there was a 20 – fold increase in VRE associated with nosocomial infections

reported by National Nosocomial Infections Surveillance (NNIS) (Katie and Carol., 2009).

There is an increasing concern regarding the presence of VRE in domestically farmed animals, which may act as reservoirs and vehicles of transmission for drug-resistant enterococci to humans, resulting in serious infections (Muriel Doufour et al., 2007). The risk of death from vancomycin – resistant enterococci (VRE) is 75 % compared with 45 % for those infected with a susceptible strains (Bearman and Wenzel., 2005).

Data on the incidence of vancomycin resistance within dairy enterococci remain controversial, through several papers that indicate very low or absence of vancomycin resistant enterococci (absence of van A and van B resistance genes) isolated from cheese (Andlrighetto et al., 2001; Jurkovic et al., 2006, Morandi et al., 2006 and Psoni et al., 2006). Presence of van A did not always indicate the resistance of enterococci to vancomycin as mentioned by Ribeiro et al. (2007), they found van A in 37% of dairy enterococci examined were susceptible to vancomycin. On the contrary, Cariolato et al. (2008) stated that all the strains harbouring the van A or van B determinants were resistant to vancomycin and showed Minimum Inhibition Concentration (MIC) values greater than 64 µg /ml. The study by Jamaly et al. (2010) showed that the absence of vancomycin resistance can be considered a positive trait for their use in food manufacture.

The objective of this study was to select sensitive vancomycin enterococcus strains which may exert beneficial effects in dairy products.

## MATERIALS AND METHODS

### *Enterococcus* species:

Studies *Enterococcus* species used were isolated and identified to species according to Albert and Anicet (1999) and secured from Dairy Department Faculty of Agriculture, Al- Azhar University.

### Trypticase Soya Agar (TSA):

Trypticase Soya Agar (TSA) (Oxoid) consist of tryptone, 17g; soya peptone, 3g; NaCl, 5g; K<sub>2</sub>HPO<sub>4</sub>, 2.5g; glucose, 2.5g; in case of TSA 15 g agar were used. Ingredients were dissolved in 1L tap water with gentle heating. The pH was adjusted to 7.0± 0.2 before sterilization at 121°C for 20 minutes.

### Sensitivity to vancomycin:

A total of 80 isolates of enterococci were tested vancomycin resistance. 77 isolates out of 80 were tested to vancomycin resistance (20  $\mu\text{g}$  and 10  $\mu\text{g}$ ) and the rest 3 isolates were tested to vancomycin resistance (40  $\mu\text{g}$ , 50  $\mu\text{g}$ , 60  $\mu\text{g}$ , 70  $\mu\text{g}$  and 80  $\mu\text{g}$ ). Overnight cultures of tested isolates were streaked onto TSA medium. Plates were left to dry about 20 to 30 min., vancomycin solution was placed in holes on surface of agar medium with sterilized pipette. The plates were incubated for 16 – 24 hr, at 37°C and then examined for zones of inhibition. Inhibition zones diameters were measured and recorded.

## RESULT AND DISCUSSION

Vancomycin resistance of tested enterococci strains isolated from different sources are shown in Tables 1 and 2. It is clear from these tables that among these cultures, 16 isolates were the most sensitive towards different vancomycin concentrations (10  $\mu\text{g}$ , up to 30  $\mu\text{g}$ ) and showed inhibition zones varied from 1.2 to 2.5cm.

On the other hand, eleven strains gave lower inhibition zones ranged from 0.6 to 1.3cm which could be considered as moderate sensitive to vancomycin.

Table (3) shows that only 18.75%, 3.75% and 3.75% of tested enterococci isolates were resistant to 10  $\mu\text{g}$ , 20  $\mu\text{g}$  and 30  $\mu\text{g}$  vancomycin, respectively, where moderate susceptibility with inhibition zones of >0.6 to <1.5cm were noticed with 72.5%, 66.25% and 23.75% by vancomycin concentrations 10  $\mu\text{g}$ , 20  $\mu\text{g}$  and 30  $\mu\text{g}$  respectively. Finally, 8.75%, 30% and 72.5% of isolated *Enterococcus* species were sensitive to 10  $\mu\text{g}$ , 20  $\mu\text{g}$  and 30  $\mu\text{g}$ , respectively.

Table (4) clearly indicates that all milk, Ras cheese and Karish cheese isolates were sensitive to vancomycin concentrations of 20  $\mu\text{g}$  and 30  $\mu\text{g}$  as compared with Domiati cheese, where 3 isolates were resistant against the same concentrations (20  $\mu\text{g}$  and 30  $\mu\text{g}$ ). Besides, approximately similar results were noticed concerning the resistance of Raw milk, Ras, Karish and Domiati cheese isolates against vancomycin concentrations of 10  $\mu\text{g}$  (3 to 4 isolates). This Table also shows that only Domiati cheese isolates (4 and 3) were resistance to both 10  $\mu\text{g}$  and 30  $\mu\text{g}$  vancomycin, respectively. Similar intermediate sensitivity reactions were observed with Raw milk, Ras and Karish cheese isolates against the three vancomycin studied concentrations being 16, 15 and 15 isolates (10  $\mu\text{g}$ ), 12, 18 and 17 isolates (20  $\mu\text{g}$ ) and 5, 7 and 6 (30  $\mu\text{g}$ ), respectively.

Enterococci isolated from Domiati cheese showed intermediate sensitivity being, 12, 6 and 1 isolates against 10  $\mu\text{g}$ , 20  $\mu\text{g}$  and 30  $\mu\text{g}$ , respectively.

However, as mentioned above, the only resistant isolated enterococci against 20  $\mu\text{g}$  and 30  $\mu\text{g}$  were found among Domiati cheese isolates being; 3 and 3 respectively. In addition, higher numbers of sensitive isolated enterococci were also detected in Domiati cheese being 4, 11 and 16 comparing with 1, 8 and 15, for Raw milk, 1, 2 and 13 for Ras cheese and 1, 3 and 14 for Karish cheese, respectively (Table 3).

Results of **Cariolato et al. (2008)** clearly indicated that the MIC values of tested *Enterococcus spp.* were greater than 64  $\mu\text{g}/\text{ml}$ . This finding was not true with the studied 80 isolates *Enterococcus* species from local sources. The MIC values of tested enterococci were above 10  $\mu\text{g}$  (Tables 1 and 2). On the other hand, Table (5) shows that the MIC values for the resistant isolates of Domiati cheese (3) were above 30  $\mu\text{g}/\text{ml}$ . This table also shows that high inhibition zones were noticed for all tested 3 isolates being 2.8 and 3cm (*E. faecalis* Dc2-2 and *E. faecalis* Dc5-2) and 3.2cm (*E. faecalis* Dc3-1).

Inhibition zones of 0.8 cm was observed with very few numbers of tested isolates (Table 6) which indicate the unpowerful effect of vancomycin concentrations of 10  $\mu\text{g}$  and 20  $\mu\text{g}$  against 11 (10  $\mu\text{g}$ ) and 6 (20  $\mu\text{g}$ ) out of the 80 enterococci isolates. In this respect **John et al. (2009)** found that only 2 out of studied 33 isolates of *E. faecium* and *E. durans* showed low level resistance to vancomycin with concentration of 8  $\mu\text{g}/\text{ml}$ .

Absence or low levels of VRE in milk and cheese isolates was previously reported by **Ortigosa et al. (2008)** and were also found by many authors in European cheese (**Teuber et al., 1999; Andrightto et al., 2001 and Jurkovic et al., 2006**). No vancomycin resistant of tested *E. faecalis* was noticed by **Gomes et al. (2008)** from Brazilian foods mainly raw milk (19 samples), pasteurized milk (11 samples) and cheeses (30 samples) comparing with only 3 (2%) *E. faecium* was found to be vancomycin resistant (32  $\mu\text{g}/\text{l}$ ) isolated from the same sources. In addition, no vancomycin resistant *E. durans* were found among these isolates from Moroccan dairy products (**Jamaly et al. 2010**). Besides all examined 68 enterococci strains belonged to *E. faecalis* (35), *E. faecium* (27) and *E. durans* (6) exhibited susceptibility to vancomycin (**Morandi et al., 2006**).

A reason of concern and contributing factor to the virulence of enterococci is their resistance against many antibiotics currently used.

Morandi et al. (2006) stated that a major concern is the emergence of vancomycin resistant enterococci, since this antibiotic is considered the last resort of treatment of multiple resistant infections. The presence of strains with potential virulence factors such as the ability to produce aggregation substances, gelatinase and haemolysin has raised a debate on the presence of enterococci in food (Franz et al., 2003).

Table (1): Vancomycin resistance of studied *Enterococcus spp* isolated from Raw milk and Ras cheese.

<i>Enterococcus species</i>	Inhibition zone/cm			<i>Enterococcus species</i>	Inhibition zone/cm		
	(10µg)	(20µg)	(30µg)		(10µg)	(20µg)	(30µg)
<i>E.durans</i> *Rm1-1	1.4	1.8	2.2	<i>E.faecalis</i> ●Rc1-1	0.6	0.8	1.2
<i>E.durans</i> Rm1-2	1.4	1.8	2.2	<i>E.faecalis</i> Rc1-2	0.8	1.0	1.5
<i>E.faecium</i> Rm2-1	1.8	2.0	2.3	<i>E.faecium</i> Rc2-1	0.8	1.0	1.4
<i>E.durans</i> Rm2-2	1.2	1.6	2.0	<i>E.faecalis</i> Rc2-2	1.0	1.2	1.7
<i>E.durans</i> Rm3-1	1.2	1.6	1.9	<i>E.faecium</i> Rc3-1	1.2	1.4	1.9
<i>E.durans</i> Rm3-2	1.0	1.4	1.8	<i>E.durans</i> Rc3-2	0.8	1.0	1.6
<i>E.faecium</i> Rm4-1	1.0	1.2	1.8	<i>E.durans</i> Rc4-1	1.0	1.2	1.6
<i>E.faecium</i> Rm4-2	1.0	1.4	1.8	<i>E.faecalis</i> Rc4-2	0.8	1.0	1.5
<i>E.faecium</i> Rm5-1	0.6	1.0	1.2	<i>E.durans</i> Rc5-1	0.8	1.2	1.8
<i>E.faecium</i> Rm5-2	0.6	1.0	1.3	<i>E.faecalis</i> Rc5-2	0.6	0.8	1.3
<i>E.faecium</i> Rm6-1	1.0	1.2	1.4	<i>E.faecalis</i> Rc6-1	1.2	1.4	1.7
<i>E.faecium</i> Rm6-2	0.6	1.0	1.4	<i>E.faecalis</i> Rc6-2	1.0	1.2	1.5
<i>E.faecium</i> Rm7-1	0.8	1.0	1.3	<i>E.faecium</i> Rc7-1	1.6	1.8	2.4
<i>E.durans</i> Rm7-2	1.4	1.8	2.2	<i>E.faecalis</i> Rc7-2	0.6	0.8	1.2
<i>E.durans</i> Rm8-1	0.8	1.0	1.7	<i>E.faecalis</i> Rc8-1	0.6	0.8	1.3
<i>E.durans</i> Rm8-2	1.4	1.8	2.3	<i>E.faecalis</i> Rc8-2	0.8	1.0	1.4
<i>E.durans</i> Rm9-1	1.0	1.2	1.6	<i>E.faecalis</i> Rc9-1	1.0	1.2	1.5
<i>E.faecalis</i> Rm9-2	1.2	1.5	2.1	<i>E.faecalis</i> Rc9-2	1.2	1.5	1.9
<i>E.faecalis</i> Rm10-1	1.0	1.2	1.5	<i>E.faecalis</i> Rc10-1	1.0	1.2	1.5
<i>E.faecalis</i> Rm10-2	1.2	1.4	1.8	<i>E.faecalis</i> Rc10-2	1.0	1.2	1.4

\*Rm =Raw milk

●Rc =Ras cheese

Table (2): Vancomycin resistance of studied *Enterococcus spp* isolated from Domiati cheese and Karish cheese.

<i>Enterococcus species</i>	Inhibition zone/cm			<i>Enterococcus species</i>	Inhibition zone/cm		
	(10µg)	(20µg)	(30µg)		(10µg)	(20µg)	(30µg)
<i>E.faecium</i> ♦Dc1-1	1.0	1.4	1.6	<i>E.durans</i> ♥K1-1	0.8	1.2	1.5
<i>E.faecalis</i> Dc1-2	1.2	1.5	1.7	<i>E.faecium</i> K1-2	0.8	1.2	1.4
<i>E.faecalis</i> Dc2-1	1.0	1.4	1.6	<i>E.faecalis</i> K2-1	1.2	1.4	1.6
<i>E.faecalis</i> Dc2-2	0.6	0.6	0.6	<i>E.durans</i> K2-2	1.0	1.2	1.5
<i>E.faecalis</i> Dc3-1	0.6	0.6	0.6	<i>E.faecium</i> K3-1	0.6	0.8	1.2
<i>E.faecalis</i> Dc3-2	1.2	1.6	2.0	<i>E.faecium</i> K3-2	0.6	1.0	1.4
<i>E.faecium</i> Dc4-1	1.2	1.5	1.7	<i>E.durans</i> K4-1	1.0	1.2	1.6
<i>E.faecium</i> Dc4-2	1.0	1.4	1.7	<i>E.durans</i> K4-2	1.3	1.5	1.8
<i>E.faecium</i> Dc5-1	1.6	1.8	2.2	<i>E.durans</i> K5-1	1.0	1.3	1.7
<i>E.faecalis</i> Dc5-2	0.6	0.6	0.6	<i>E.durans</i> K5-2	0.6	0.8	1.2
<i>E.durans</i> Dc6-1	1.8	2.2	2.5	<i>E.durans</i> K6-1	1.6	1.8	2.0
<i>E.durans</i> Dc6-2	1.2	1.6	1.8	<i>E.faecium</i> K6-2	0.8	1.2	1.4
<i>E.faecium</i> Dc7-1	1.4	1.8	2.0	<i>E.faecalis</i> K7-1	1.0	1.3	1.6
<i>E.faecium</i> Dc7-2	1.2	1.6	1.9	<i>E.durans</i> K7-2	1.2	1.4	1.8
<i>E.faecium</i> Dc8-1	1.2	1.6	2.0	<i>E.durans</i> K8-1	1.0	1.2	1.6
<i>E.faecium</i> Dc8-2	1.0	1.4	1.8	<i>E.faecium</i> K8-2	0.6	1.0	1.3
<i>E.durans</i> Dc9-1	1.8	2.2	2.4	<i>E.durans</i> K9-1	1.2	1.6	1.8
<i>E.durans</i> Dc9-2	0.6	1.0	1.3	<i>E.durans</i> K9-2	1.2	1.4	1.6
<i>E.durans</i> Dc10-1	1.6	2.0	2.3	<i>E.durans</i> K10-1	1.0	1.2	1.5
<i>E.faecium</i> Dc10-2	1.0	1.2	1.6	<i>E.faecalis</i> K10-2	1.0	1.2	1.6

♦Dc =Domiati cheese

♥K =Karish cheese

Table (3): Number and percentage of susceptible (S), intermediate (M), and resistant (R) Enterococci strains isolated from different sources against studied vancomycin concentrations.

Vancomycin concentrations	Reactions					
	R		M		S	
	No	%	No	%	No	%
(10µg)	15	18.75	58	72.5	7	8.75
(20µg)	3	3.75	53	66.25	24	30
(30µg)	3	3.75	19	23.75	58	72.5

R= Resistant (inhibition zone = 0.6 cm)

M= Intermediate sensitivity (inhibition zone > 0.6

S = Susceptible (inhibition zone ≥ 1.5 cm)

Table (4): Susceptibility of Enterococci strains isolated from different sources against different vancomycin concentrations.

Vancomycin concentration	Raw milk (20 isolates)			Ras cheese (20 isolates)			Karish cheese (20 isolates)			Domati cheese (20 isolates)		
	R	M	S	R	M	S	R	M	S	R	M	S
(10µg)	3	16	1	4	15	1	4	15	1	4	12	4
(20µg)	0	12	8	0	18	2	0	17	3	3	6	11
(30µg)	0	5	15	0	7	13	0	6	14	3	1	16

R= Resistant (inhibition zone = 0.6 cm)

M= Intermediate sensitivity (inhibition zone > 0.6

S= Susceptible (inhibition zone ≥1.5 cm)

Table (5): Effect of increasing vancomycin concentrations on the three resistance isolates.

<i>Enterococcus species</i>	Inhibition zone/cm					
	(30µg)	(40µg)	(50µg)	(60µg)	(70µg)	(80µg)
<i>E.faecalis</i> ♦Dc2-2	0.6	2.3	2.7	2.8	2.8	2.8
<i>E.faecalis</i> Dc3-1	0.6	2.8	2.9	3.0	3.0	3.2
<i>E.faecalis</i> Dc5-2	0.6	2.4	2.6	2.9	3.0	3.0

♦Dc =Dommati cheese

Table (6): Sensitivity of Enterococci strains isolated from different sources against very low vancomycin concentrations.

Samples	Inhibition zone/cm							
	(10µg)				(20µg)			
	0.6	0.8	1	>1	0.6	0.8	1	>1
Raw milk	3	2	6	9	0.0	0.0	5	15
Ras cheese	4	6	6	4	0.0	4	5	11
Dommati cheese	4	0.0	5	11	3	0.0	1	16
Karish cheese	4	3	7	6	0.0	2	2	16
Total	15	11	24	30	3	6	13	58



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## الملخص العربي

مقاومة بعض أنواع *Enterococcus* المعزولة من اللبن الخام وبعض الجبن للمضاد

الحيوي فانكوميسين

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تم دراسة حساسية سلالات enterococci المعزولة ضد تركيزات مختلفة من vancomycin (١٠ ميكروجرام ، ٢٠ ميكروجرام ، ٣٠ ميكروجرام ، ٨٠ ميكروجرام) حيث كانت ١٨,٧٥ % ، ٣,٧٥ % ، ٣,٧٥ % من عزلات enterococci المختبرة مقاومة لتركيزات vancomycin ١٠ ميكروجرام ، ٢٠ ميكروجرام ، ٣٠ ميكروجرام على التوالي، حيث تم ملاحظة الحساسية المتوسطة ( ذو مناطق تثبيط أكبر من ٠,٦ سم لأقل من ١,٥ سم) في ٧٢,٥ % ، ٦٦,٢٥ % ، ٢٣,٧٥ % من العزلات باستخدام تركيزات vancomycin ١٠ ميكروجرام ، ٢٠ ميكروجرام ، ٣٠ ميكروجرام على التوالي. أخيرا ٨,٧٥ % ، ٣٠ % ، ٧٢,٥ % لأنواع *Enterococcus* المعزولة حساسة للتركيزات المختلفة وهي ١٠ ميكروجرام ، ٢٠ ميكروجرام ، ٣٠ ميكروجرام على التوالي. لقد وجد أن كل عزلات اللبن الخام والجبن القريش والجبن الراس حساسة لتركيزات ٢٠ ميكروجرام ، ٣٠ ميكروجرام مقارنة بالجبن الاديمياتى حيث ٣ عزلات أظهرت مقاومة ضد تركيزات ٢٠ ميكروجرام ، ٣٠ ميكروجرام. كان قطر المنطقة الخالية من النمو البكتيرى ٠,٨ سم فى أعداد قليلة جدا من العزلات المختبرة وذلك يوضح التأثير الغير قوى لتركيز ١٠ ميكروجرام ضد (١١ عزله من ٨٠ عزله) ، كذلك تركيز ٢٠ ميكروجرام ضد (٦ عزلات من ٨٠ عزله).

أكبر مناطق تثبيط عند التركيز الأعلى من المضاد الحيوي vancomycin (٦٠ ميكروجرام ، ٧٠ ميكروجرام) لوحظ فى السلالتين *E. faecalis* Dc2-2 ، *E. faecalis* Dc5-2 ، بينما كان ذلك عند تركيز ٨٠ ميكروجرام فى السلالة *E. faecalis* Dc3-1. أظهرت النتائج أيضا أن هناك ١٦ سلالة كانت أكثر حساسية بمناطق تثبيط ٢ سم أو أكثر وأن هناك ١١ سلالة يمكن اعتبارها ذات حساسية متوسطة تجاه هذا المضاد الحيوي (١,٢ أو ١,٣ سم).

