PHARMACODYNAMICS OF AVILAMYCIN TOWARDS STRAINS OF *CLOSTRIDIUM PERFRINGENS* ISOLATED FROM EPIZOOTIC RABBIT ENTEROPATHY

Simjee S.[1]*, Heffron A.L.[1], Agnoletti F.[2], Pridmore A.[3], Pradella G.[4]

[1]Elanco Animal Health, Lilly House, Priestley Road, Basingstoke, RG24 9NL, England
[2]Istituto Zooprofilattico Sperimentale delle Venezie, Viale Brigata Treviso 13/a, 31100 Treviso, Italy
[3]Don Whitley Scientific Limited, 14 Otley Road, Shipley, West Yorkshire, BD17 7SE, England
[4]Eli Lilly Italia S.p.A., Divisione Elanco Animal Health, Via Gramsci 733, Sesto Fiorentino (FI), Italy
*Corresponding author: simjeess@lilly.com

ABSTRACT

For treatment of outbreaks of epizootic rabbit enteropathy syndrome, where *Clostridium perfringens* is frequently isolated, avilamycin should be administered for 4 weeks at the dose of 7 mg/kg of body weight. This corresponds to approximately 80 ppm in the complete feed. A total of 89 clinical isolates of *C. perfringens* were recovered from diseased rabbits in Italy, France and Spain. The MIC_{90} values for these clinical isolates were between 0.5 and 1 µg/ml. There was no noticeable MIC variation in the *C. perfringens* recovered from the three countries. In the absence of clinical breakpoints for avilamycin against *C. perfringens* a wild-type cut-off value of ≤2µg/ml has been proposed. The minimum bactericidal concentration (MBC) of avilamycin against 10 *C. perfringens* strains was determined and ranged from 2->256 µg/ml. The Post Antibiotic Effect (PAE) and Post Antibiotic Sub-MIC effect (PA SME) exerted by avilamycin against two *C. perfringens* strains was investigated. After exposure to avilamycin at 5×MIC, PAEs and PA SMEs were demonstrated against both *C. perfringens* strains. The magnitude increased with subsequent increasing avilamycin concentrations. Growth of each strain was also inhibited in a dose-dependent manner by exposure to sub-MIC concentrations of avilamycin without initial exposure to a higher concentration. The sub-MIC effects were also observed against each strain. Prolonged exposure to sub-MIC concentrations of avilamycin exerted a greater overall effect than short (2 h) exposure to a high concentration (5×MIC). In a series of kill kinetic studies against 2 *C. perfringens* strains, against one strain the avilamycin activity was time-dependent but was additionally concentration dependent because bactericidal activity was achieved at the highest concentration tested. In the second strain avilamycin activity at concentrations above the MIC was bacteriostatic, essentially time-dependent and influenced very little by concentration.

Keywords: *Clostridium perfringens*, Avilamycin, Minimum Inhibitory Concentration (MIC), Epizootic Rabbit Enteropathy.

INTRODUCTION

The digestive disease is the main cause of mortality in industrial fattening rabbit farms. Two main digestive syndromes are generally identified: colibacillosis and Epizootic Rabbit Enteropathy (ERE). Digestive disease is rarely caused by one single pathogen. It is quite frequent that there is an overlapping of different causative agents that work together to develop the digestive syndrome (Marlier *et al.*, 2006). In a recent publication (Morel-Saives *et al.*, 2007), the economical impact of an episode of digestive disease was evaluated to be 0.78 € by produced rabbit. This cost allowed us to look for a therapeutic strategy.

Avilamycin is an antibiotic obtained by fermentation of a strain of *Streptomyces viridichromogenes*. This compound is active against Gram positive bacteria, including *Clostridium perfringens*. Recently,

avilamycin has been used by rabbit producers in Italy as a new option to control digestive syndrome associated with *Cl. perfringens*.

The objective of this study was to evaluate the pharmacodynamics parameters of avilamycin against clinical cases where strains of *Cl. perfringens* were recovered from rabbits in Italy, France and Spain.

MATERIALS AND METHODS

Minimum Inhibitory Concentration (MIC)

MIC is defined as the lowest concentration of antimicrobial substance which, under defined *in vitro* conditions, prevents the growth of bacteria. First and foremost it should be noted that currently there are no CLSI (previously NCCLS) interpretive criteria available for avilamycin.

A total of 89 strains of *Cl. perfringens* recovered from rabbits with epizootic rabbit enteropathy in Italy (n = 60), France (n = 19) and Spain (n = 10). The strains were collected from diverse regions of each country, geographically distant farms and not from the same outbreak, as recommended by the CVMP627 guidelines. A total of 85 strains were collected between 2003 and 2004 and 4 strains were collected in the latter part of 2002. Antibiotic dilutions and susceptibility testing were done as described in CLSI M7-A6 and M11-A6 documents. Testing methodology followed the recommendations for anaerobe testing as described in the CLSI M7-A6 and M11-A6 documents, using *Cl. perfringens* ATCC 13124 as quality control strain.

Minimal Bactericidal Concentration (MBC)

The Minimum bactericidal concentration (MBC) is the lowest concentration of an antimicrobial substance which, under defined *in vitro* conditions, reduces bacterial counts by 99.9% within a fixed time period (generally 24 hours).

The MBC of avilamycin against 10 *Cl. perfringens* strains recovered from rabbits was determined. The test system in this study phase used standardised broth microdilution MIC/MBC methodology, essentially as described by the CLSI (NCCLS, 1999) but with modifications to the culture media and incubation conditions to allow growth of anaerobic bacteria.

Kinetics of bacterial killing

The kinetics of killing is highly relevant to predicting *in vivo* activity of the test compound. The rates of bacterial killing (antibacterial kill kinetics) produced by avilamycin against two strains of *Cl. perfringens* isolated from rabbits were investigated. Antibacterial kill kinetics was determined using Wilkins-Chalgren Anaerobe Broth (WCAB) in shake flask cultures. This methodology was based on that published by the CLSI in document M26-A, but with appropriate modifications (use of WCAB and anaerobic incubation) for testing anaerobic bacteria. Antibacterial kill kinetics was determined for avilamycin concentrations equivalent to 2, 4, 8, 16 and 32 times the MIC, using a time-kill method with shake flask cultures. For each bacterial strain, inoculum density at test initiation was consistent between experiments and approached 10^6 cfu/ml.

Post-antibiotic effect (PAE)

I*n vitro* post-antibiotic effect (PAE) is defined as the period of suppression of bacterial growth after short exposure of organisms to an antimicrobial. Using bacterial counts the PAE is calculated as: PAE=T–C , where T is the time required for the bacterial counts of the exposed cultures to increase one $\log_{10}$ above the counts observed immediately after washing/dilution and C the corresponding time required for the counts of the untreated cultures.

Standardised study protocols follow the general procedure of inoculating cells into growth medium containing the test antibiotic. After a fixed exposure period, often 2 hours, the test antibiotic is removed from the culture and the time required for surviving bacteria to resume growth is determined by monitoring the change in the concentration of cfu/ml, using an untreated control as a reference point. The difference in time after which the cfu/ml increases by one $\log_{10}$ in the resulting growth curves is the PAE.

This part of the study investigated both PAEs and PA Sub-MIC Effect (PA SMEs) exerted by avilamycin against two *Cl. perfringens* strains isolated from rabbits.

Anaerobic suspensions of each strain (10^6 cfu/ml in Wilkins-Chalgren Anaerobe Broth) were initially exposed to avilamycin at 5× the respective MIC; an antibiotic-free control was also prepared. After 2 h anaerobic incubation at 35±1°C, bacterial suspensions were centrifuged and washed twice under anaerobic conditions. Washed cells were resuspended in antibiotic-free growth medium and in media containing avilamycin at 0.25×MIC and 0.5×MIC for each strain. All preparations were incubated anaerobically at 35±1°C for 24±1 h and viable bacterial counts were determined hourly during the first 12 h. PAEs produced by brief exposure of bacteria to avilamycin at 5×MIC and subsequent exposure to sub-MIC concentrations were calculated using the equation "PAE=T–C".

RESULTS AND DISCUSSION

Minimum Inhibitory Concentration (MIC)

The MIC distribution is shown in Table 1 and individual country and summary results are summarised in Table 2 below as MIC_{50}, MIC_{90}, geometric mean and range. In the absence of clinical breakpoints the MIC distribution supports a wild-type cut-off value of ≤2 µg/ml and based on this value all of the isolates were susceptible to avilamycin.

Table 1: Summary of the avilamycin MIC distribution for *Cl. perfringens* strains isolated from clinical cases

Country	MIC (µg/ml)				
	0.063	0.125	0.25	0.5	1
Italy	3	10	23	22	2
France	-	2	8	6	3
Spain	-	2	5	3	-

Table 2: Summary of avilamycin MIC data for *Cl. perfringens* strains isolated from clinical cases

Country	MIC (µg/ml)				
	Number of Strains	MIC_{50}	MIC_{90}	GeometricMean	MIC Range
Italy	60	0.25	0.5	0.28	0.063-1
France	19	0.25	1	0.36	0.125-1
Spain	10	0.25	0.5	0.31	0.125-0.5

Minimal Bactericidal Concentration (MBC)

The avilamycin MBCs are shown in Table 3. For 4 of the *Cl. perfringens* strains the MBC was between 2 and 32 times the MIC. For 2 *Cl. perfringens* strains (strains LH84600 and 481/4/R03) the MBC was calculated to be 128 µg/ml and 256 µg/ml. For the remaining 4 *Cl. perfringens* strains the MBCs was >256 µg/ml. Reproducibility of the test system was confirmed by the consistent MIC results obtained for the internally selected *Cl. perfringens* reference strain.

Kinetics of bacterial killing

An appraisal of the 4 kill kinetic experiments conducted for each *Cl. perfringens* strain demonstrates that against strain DWC 13136, no bactericidal activity was achieved at avilamycin concentrations up

to 32×MIC, but inhibition of bacterial growth was observed. At avilamycin concentrations equivalent to 8×MIC and above, this inhibition was maintained throughout the 24 h test period. Thus, avilamycin activity against DWC 13136 was bacteriostatic.

Table 3: Summary of avilamycin antibacterial activity (MIC and MBC) against strains of *Cl. perfringens* isolated from clinical cases in rabbits

Cl. perfringens strain number	Avilamycin MIC (µg/ml)	Avilamycin MBC (µg/ml)
LH84717	0.5	2
LH84600	0.25	256
GP0237	0.5	8
L96	0.5	16
GP0132	0.25	>256
416/2/04UD	0.5	1
562/1/R05	1	>256
481/4/R03	0.25	128
741/2/R05	0.25	>256
LH84732	0.5	>256

Against strain DWC 13137, temporary inhibition of growth was achieved at avilamycin concentrations up to 8×MIC, but higher concentrations produced true bactericidal activity: consistent bactericidal activity was achieved at a concentration equivalent to 32×MIC.

Thus, the antibacterial activity of avilamycin against *Cl. perfringens* isolates from rabbits varied between the two isolates tested in the present study. Against strain DWC 13136, activity at concentrations above the MIC was bacteriostatic, essentially time-dependent and influenced very little by concentration. Against strain DWC 13137, activity was also time-dependent but was additionally concentration dependent because bactericidal activity was achieved at the highest concentration tested.

Post-antibiotic effect (PAE)

These results are summarised in Table 4.

Table 4: Summary of the PAE studies against strains of *Cl. perfringens* isolated from clinical cases in rabbits

Cl. perfringens strain	Avilamycin MIC (µg/ml)	Replicate Test	Mean PAE or PA SME (h) at each avilamycin concentration		
			No Antibiotic	0.25×MIC	0.5×MIC
DWC 13136	0.25	1	1.3	2.9	4.9
		2	1.8	4.8	8.2
DWC 13137	1	1	0.7	1.7	6.8
		2	1.4	3.4	3.5

Thus, after exposure to avilamycin at 5×MIC, PAEs and PA SMEs were demonstrated against both *Cl. perfringens* strains. The magnitude of these effects increased with secondary avilamycin concentration. Growth of each strain was also inhibited in a dose-dependent manner by exposure to sub-MIC concentrations of avilamycin without initial exposure to a higher concentration (Table 5).

Table 5: Summary of the PA-SME studies against strains of *Cl. perfringens* isolated from clinical cases in rabbits

Cl. perfringens strain	Avilamycin MIC (µg/ml)	Replicate Test	Mean sub-MIC effect (h) at each avilamycin concentration	
			0.25×MIC	0.5×MIC
DWC 13136	0.25	1	1.3	3.2
		2	2.4	5.7
DWC 13137	1	1	0.2	3.0
		2	1.9	2.4

Thus, sub-MIC effects were also observed against each strain. Furthermore, comparison of PAEs, PA SMEs and SMEs suggests that SMEs represented a significant component of avilamycin's inhibitory

activity against *Cl. perfringens*. Specifically, prolonged exposure to sub-MIC concentrations of avilamycin exerted a greater overall effect than short (2 h) exposure to a high concentration (5×MIC).

As the antibiotic concentration may fluctuates between treatment periods and the amount of feed intake by the rabbit, the prolonged PAE and PA SME ensures sufficient exposure of the *Cl. perfringens* to avilamycin.

CONCLUSIONS

The data obtained in this study clearly suggest a high predictive efficacy for avilamycin when dosed at 80 ppm in feed to rabbits against *Cl. perfringens*. In the absence of clinical breakpoints a wild-type cut-off value of ≤2µg/ml has been proposed. Based on this wild-type cut-off value, there was no resistance in *Clostridium perfringens* to avilamycin in the 89 clinical strains recovered from France, Italy and Spain.

REFERENCES

Marlier D., Dewrée R., Lassence C., Licois D., Mainil J., Coudert P., Meulemans L., Ducatelle R., Vindevogel H. 2006. Infectious agents associated with epizootic rabbit enteropathy: isolation and attemps to reproduce the syndrome. *Vet. J., 172 (3), 493-500.*

Morel-Saives A., Limet A. 2007. Assessment of the technical and economical impact of digestive disease in fattening rabbit. Interest of treatment strategy. *In: Proc. 12èmes Journées de Recherche Cunicole, 2007 November, Le Mans, France.*

NCCLS. Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard – Sixth Edition (January 2004). NCCLS document M11-A6. *NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA 2002*.

NCCLS. Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters for Veterinary Antimicrobial Agents; Approved Guideline - Second Edition. NCCLS document M37-A2. *NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA, 2002*.

NCCLS. Methods for determining bacterial activity of antimicrobial agents: Approved guideline NCCLS document M26-A. *NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA, 1999.*

NCCLS. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard – Sixth Edition (January 2003). NCCLS document M7-A6. *NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA, 2002*.

NCCLS. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Informational Supplement. NCCLS document M31-S1. *NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA, 2004*.