The University of Nottingham

OTITIS MEDIA WITH EFFUSION: CURRENT TREATMENT, NEW UNDERSTANDING OF ITS AETIOPATHOGENESIS, AND A NOVEL THERAPEUTIC APPROACH

Matija Daniel

BMedSc(Hons) MBChB(Hons) CertMgmt(Open) PGDipMedEd FHEA FRCS FRCSEd(ORL-HNS)

Thesis submitted to the University of Nottingham

for the Degree of Doctor of Philosophy

JULY 2013

BIOFILMS WITH ANTIBIOTICS

9.1 INTRODUCTION

Work presented in this thesis and previous published literature points to a clear deficiency in the current treatment for OME, with approximately 25% of children having to have grommets (ventilation tube, VT) surgery more than once. With the realisation over the last few years that biofilms are implicated in the aetiology of OME, the high recurrence rate is not surprising, as it is likely that VT insertion merely removes the effusion that is a product of inflamed middle ear mucosa, but the underlying biofilm is not addressed, and may simply re-grow in the future once the VT has extruded.

The previous section has detailed the development of an *in vitro* biofilm model that could ultimately be used to test a modified release antibiotic pellet for use in the middle ear in children with OME with the aim of improving current treatment. However, prior to testing the modified release pellets, we wished to test whether antibiotics (as a solution) can actually eradicate biofilms in this setting.

Previous published work suggests that biofilm eradication with antibiotics is possible, but the antibiotic concentration required is thought to be in the region of 100 to 1000 times higher than the level needed to inhibit planktonic bacteria, the MIC (Donlan and Costerton 2002) (Vorachit, et al. 1993) (Nickel,

et al. 1985) (Ceri, et al. 1999) (Olson, et al. 2002). We therefore set out to assess the effect of a range of antibiotic concentrations on *S aureus* biofilms grown on silicone discs in the *in vitro* model describe above, to establish if biofilm eradication using antibiotics is possible. A crucial part of experiment planning was confirmation that a biofilm is eradicated rather than just inhibited, by allowing a 5 day "resuscitation" period, where the antibiotic is removed at the end of the treatment period and any bacteria provided with optimum growing conditions prior to sampling to see if any bacteria re-grow once the inhibitory effect of antibiotics is removed.

9.2 METHODS

The model established above was used to test antibiotics against biofilms. Antibiotic solutions were used, with bacteria sampled at set time intervals as detailed in the model. The discs were trypsinised for 15 min and sonicated for 5 (as deemed optimal in the "model development" experiments) prior to plating the solution on sheep blood agar (Oxoid, Cambridge UK). Discs with biofilm were analysed in triplicate.

Biofilms were allowed a set time to develop (1, 5, 14 and 21 days), and then antibiotics (rifampicin (Sigma-Aldrich, Gillingham, UK) and clindamycin (Sigma-Aldrich, Gillingham, UK), were added with concentrations ranging from 1xMIC (inoculum was tested using the Etest strip) to 10,000xMIC (Table 9.2.1). The antibiotic solution was used in these experiments in order to ascertain the likely antibiotic concentrations required by the modified release pellets in later experiments.

MIC	RIFAMPICIN µg/mL	CLINDAMYCIN µg/mL
x1	0.006	0.1
x10	0.06	1
x100	0.6	10
x1000	6	100
x10000	60	1000

Table 9.2.1: Antibiotic concentrations in biofilm models

Importantly, evidence of biofilm eradication, rather than just inhibition was sought. Biofilms may be inhibited by the presence of antibiotics, but the key to eradication is demonstration that biofilms do not re-grow once antibiotics are

removed and any bacteria are provided with good growing conditions (resuscitated). In the case of one day old biofilms, the disc used for resuscitation was the same disc that was sonicated for purposes of bacterial counting; after sonication and plating of solution, the discs were kept in a fridge overnight, and those that proved negative were then placed into fresh TSB for five days. However, as there was a concern that sonication may possibly reduce the number of bacteria on the discs, when analysing 5, 14 and 21 day old biofilms we switched to using fresh rather than previously sonicated discs in resuscitation experiments.

9.3 RESULTS

The results of biofilm eradication experiments using antibiotics are shown in graph format. The graphs present the number of colony forming units (CFU) per mL, on a logarithmic scale, derived from different discs. Planktonic bacteria are shown in blue, biofilm bacteria on silicone discs in red. Control silicone discs without bacteria contained no bacteria. Where resuscitation of discs was undertaken, results are shown on the graphs as resuscitation culture positive (RP) or resuscitation culture negative (RN).

In many experiments the LiveDead stain was performed in addition to culture. Both may be expected to give the same result as they both look for live bacteria, and indeed a comparison of the two techniques shows that the agreement between the two is substantial. Raw data on the two techniques is shown in this chapter, but the analysis and interpretation is considered as a part of the development of the *in vitro* model shown in Chapter 8.

The experiments were carried out on two different strains of *Staph aureus* (our laboratory reference F2387 and F2315), with two strain used to confirm applicability of results.

9.3.1 Eradication of one day old biofilms

Treatment of one day old biofilms for one day

Exposure to antibiotics for 1 day failed to inhibit biofilms even at 10,000xMIC (Fig 9.3.1.1).

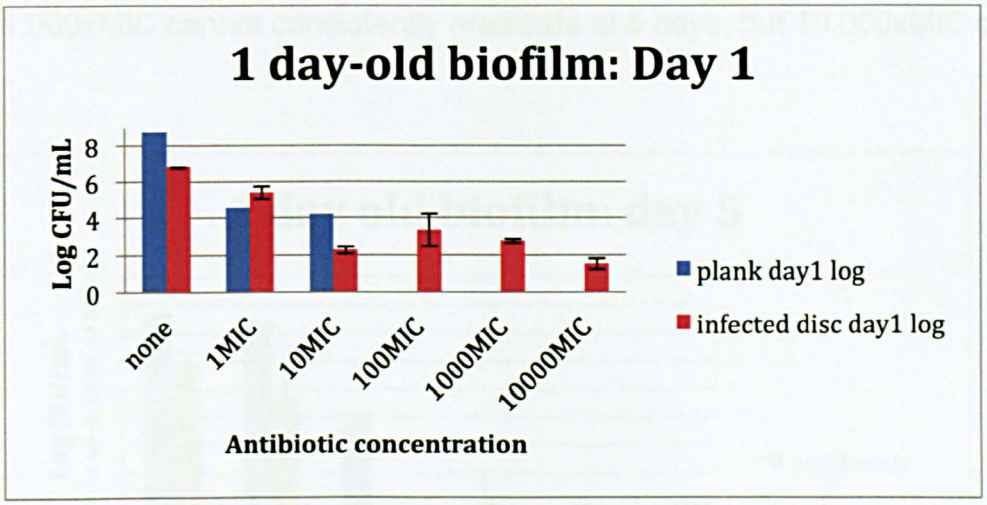


Fig 9.3.1.1: One day old biofilms treated with antibiotics for one day (*S aureus* F2387). Clean control discs contained no bacteria. Error bars indicate standard deviation.

Treatment of one day old biofilms for five days

At 5 days exposure, biofilms are inhibited by 1,000x and 10,000xMIC (Fig 9.3.1.2). Those discs from 1,000x and 10,000xMIC were resuscitated by incubation in fresh TSB for 5 days. One of the three 1000xMIC discs contained 15000 CFU/mL, the other two were culture negative, as were all three 10,000xMIC discs and clean controls (Table 9.3.1.1). This suggests that 1,000xMIC cannot consistently eradicate at 5 days, but 10,000xMIC can.

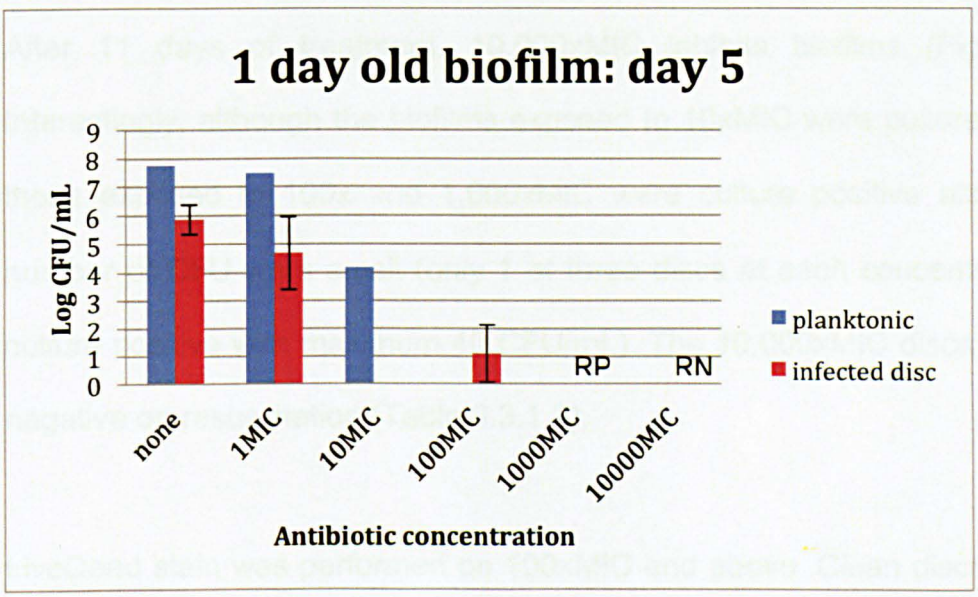


Fig 9.3.1.2: One day old biofilms treated with antibiotics for five days (*S aureus* F2387). RP: resuscitation culture positive; RN: resuscitation culture negative. Clean control discs contained no bacteria. Error bars indicate standard deviation.

xMIC	Disc1 CFU / mL		Disc2 CFU / mL		Disc3 CFU / mL	
	In antibiotics	Resuscitated	In antibiotics	Resuscitated	In antibiotics	Resuscitated
1,000	0	15000	0	0	0	0
10,000	0	0	0	0	0	0

Table 9.3.1.1: Resuscitation of one day old biofilms (*S aureus* F2387) treated with antibiotics for five days. Numbers refer to CFU/mL.

Treatment of one day old biofilms for 11 days

After 11 days of treatment, 10,000xMIC inhibits biofilms (Fig 9.3.1.3). Interestingly, although the biofilms exposed to 10xMIC were culture negative, those exposed to 100x and 1,000xMIC were culture positive although the number of CFU were small (only 1 of three discs at each concentration was culture positive with maximum 40 CFU/mL). The 10,000xMIC discs were also negative on resuscitation (Table 9.3.1.2).

LiveDead stain was performed on 100xMIC and above. Clean discs and PBS control contained no bacteria. No-antibiotic control contained live bacteria. One of the three 100xMIC discs contained live bacteria (culture was 30 CFU/mL), but the remaining 100xMIC discs and other biofilms exposed to 1,000 and 10,000xMIC contained only dead bacteria (Fig 9.3.1.4).

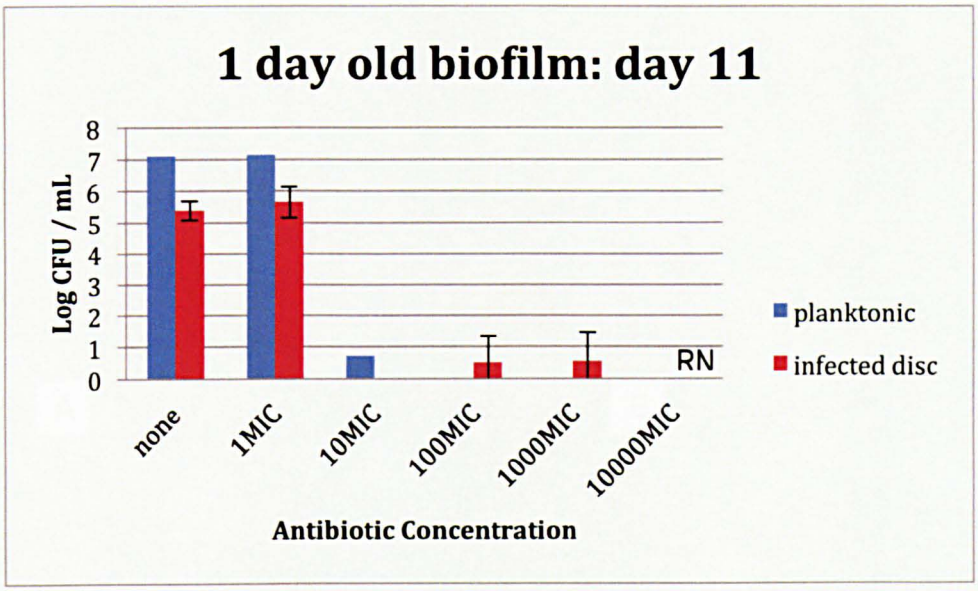


Fig 9.3.1.3: One day old biofilms treated with antibiotics for 11 days (*S aureus* F2387). RN: resuscitation culture negative. Clean control discs contained no bacteria. Error bars indicate standard deviation.

xMIC	Disc1 CFU / mL		Disc2 CFU / mL		Disc3 CFU / mL	
	In antibiotics	Resuscitated	In antibiotics	Resuscitated	In antibiotics	Resuscitated
100	0 N	0 N	30 L	0 N	0 N	0 N
1,000	40 N	85 N	0 N	0 N	0 N	0 N
10,000	0 N	0 N	0 N	0 N	0 N	0 N

Table 9.3.1.2: Resuscitation of one day old biofilms (*S aureus* F2387) treated with antibiotics for 11 days. Table incorporates results from the LiveDead stain (L: live bacteria; N: LiveDead stain contains no bacteria or dead bacteria only). Numbers refer to CFU/mL.

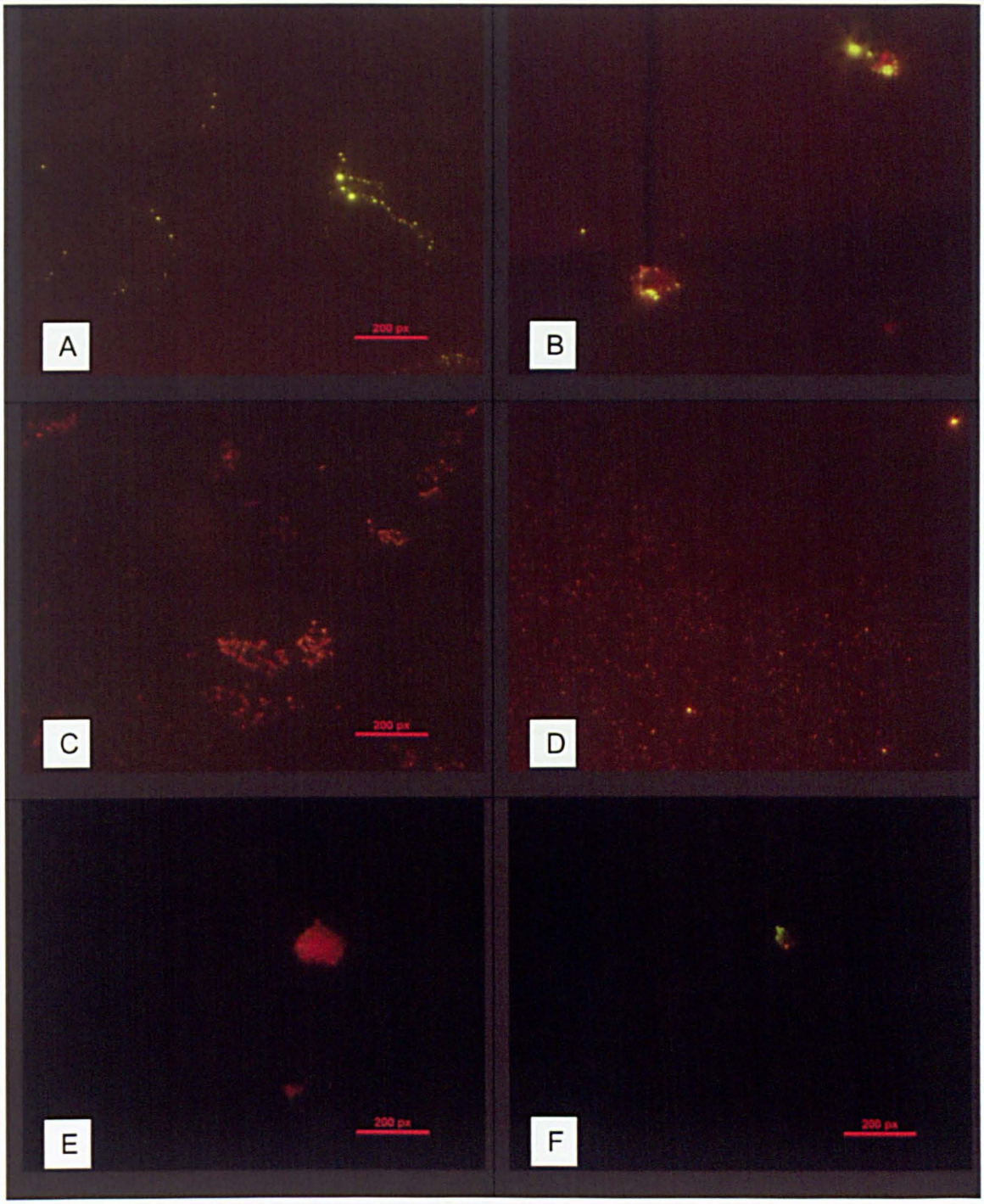


Fig 9.3.1.4 LiveDead stain of one day old biofilms (*S aureus* F2387) treated with antibiotics for 11 days. A, B: Live bacteria from no-antibiotic control biofilm discs. C: dead bacteria from 100xMIC biofilm disc 1. D: dead bacteria from 1,000xMIC disc 1. E: dead bacteria from 10,000xMIC disc 1. F: Live bacteria from 100xMIC disc 2.

Treatment of one day old biofilms for 19 days

After 19 days of treatment, the infected discs were culture negative at 10xMIC and above (Fig 9.3.1.5). However, on resuscitation the 10xMIC disc biofilms re-grew (Table 9.3.1.3), and the LiveDead stain showed live bacteria in 10xMIC discs and 100xMIC discs (Fig 9.3.1.6).

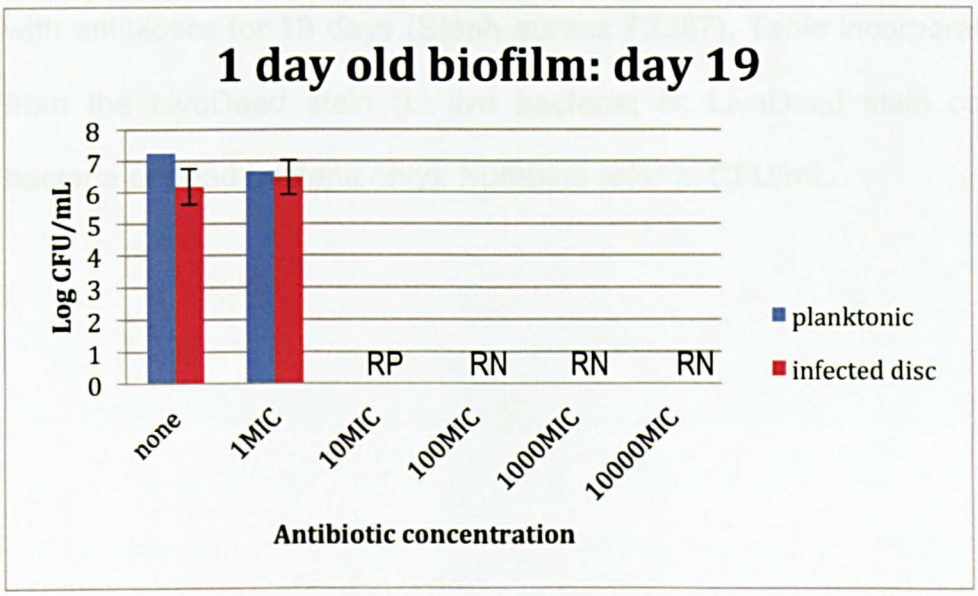


Fig 9.3.1.5: One day old biofilms treated for 19 days (*S aureus* F2387). RP: resuscitation culture positive; RN: resuscitation culture negative. Clean control discs contained no bacteria. Error bars indicate standard deviation.

xMIC	Disc1 CFU / mL		Disc2 CFU / mL		Disc3 CFU / mL	
	In antibiotics	Resuscitated	In antibiotics	Resuscitated	In antibiotics	Resuscitated
10	0	60000	0	70000	0	0
100	0 P	0 N	0 P	0 N	0 N	0 N
1,000	0 N	0 N	0 N	0 N	0 N	0 N
10,000	0 N	0 N	0 N	0 N	0 N	0 N

Table 9.3.1.3: Resuscitation of one day old biofilms (*S aureus* F2387) treated with antibiotics for 19 days (Staph aureus F2387). Table incorporates results from the LiveDead stain (L: live bacteria; N: LiveDead stain contains no bacteria or dead bacteria only). Numbers refer to CFU/mL.

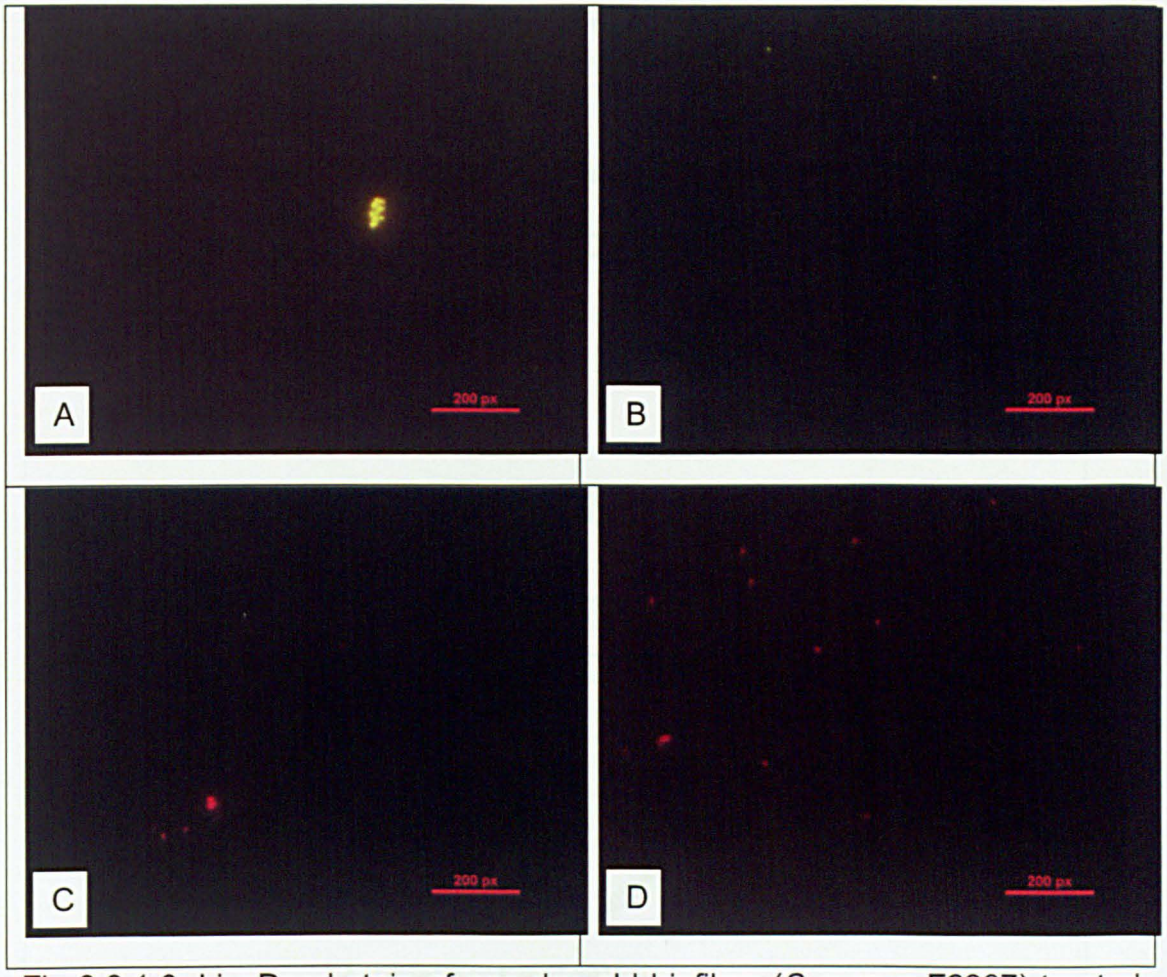


Fig 9.3.1.6: LiveDead stain of one day old biofilms (*S aureus* F2387) treated with antibiotics for 19 days. A: Clump of live bacteria from 10xMIC. B: Live bacteria from 100xMIC. C: Dead bacteria from 100xMIC. D: Dead bacteria from 1000xMIC.

9.3.2 Eradication of five day old biofilms (*S aureus* F2387)

Treatment of five day old biofilms for one day

After 1 day of treatment, not even 10,000xMIC was able to inhibit *S aureus* F2387 biofilms (Fig 9.3.2.1).

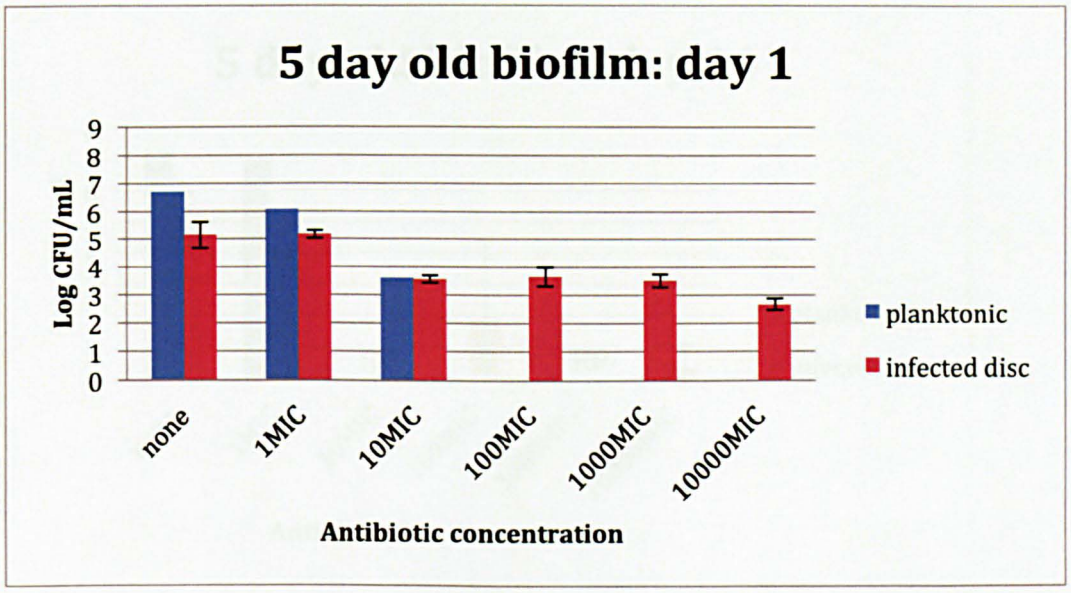


Fig 9.3.2.1: Five day old biofilms (*S aureus* F2387) treated with antibiotics for one day. Clean control discs contained no bacteria. Error bars indicate standard deviation.

Treatment of five day old biofilms for 14 days

After 14 days, 10x and 1,000xMIC inhibited bacteria (Fig 9.3.2.2), but 10,000xMIC did not (although the count was only 15 CFU/mL). However, both the 10x and 1,000xMIC were positive on resuscitation, whereas in fact the 10,000xMIC was negative on resuscitation (Table 9.3.2.1).

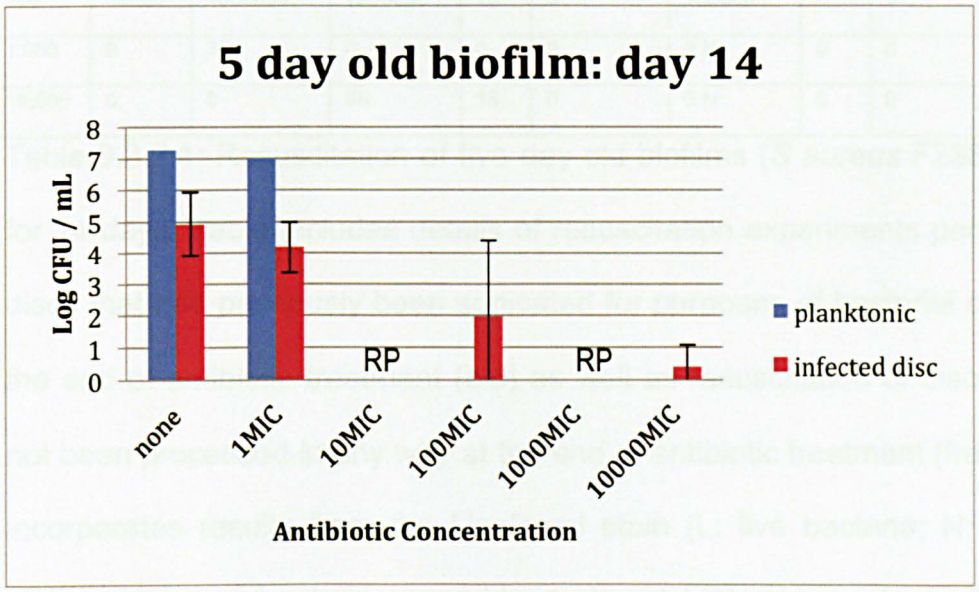


Fig 9.3.2.2: Treatment of five day old biofilms (*S aureus* F2387) with antibiotics for 14 days. RP: resuscitation culture positive. Clean control discs contained no bacteria. Error bars indicate standard deviation.

For resuscitation testing in this particular case, both the original previously sonicated disc (sonicated to obtain bacterial counts), as well as a fresh disc that had not been sonicated before were resuscitated; the data shown at 14 and 21 days (next) shows that the fresh discs yielded more bacteria than those previously processed, and therefore fresh discs were used for further experiments (Thus only those experiments performed on one day old biofilms

had resuscitation data that only refers to resuscitation of discs that had been previously sonicated).

xMIC	Disc1 CFU / mL			Disc2 CFU / mL			Disc3 CFU / mL		
	In abx	Resuscitated (old)	Resuscitated (fresh)	In abx	Resuscitated (old)	Resuscitated (fresh)	In abx	Resuscitated (old)	Resuscitated (fresh)
10	0	0	100000L	0	0	100000 L	0	0	1000000L
100	50000	1000000	100000L	15	0	100000 L	0	0	100000L
1,000	0	35	0L	0	0	0 N	0	0	10000L
10,000	0	0	0N	15	0	0 N	0	0	0 N

Table 9.3.2.1: Resuscitation of five day old biofilms (*S aureus* F2387) treated for 14 days. Table includes details of resuscitation experiments performed on discs that had previously been sonicated for purposes of bacterial counting at the end of antibiotic treatment (old) as well as resuscitation of discs that had not been processed in any way at the end of antibiotic treatment (fresh). Table incorporates results from the LiveDead stain (L: live bacteria; N: LiveDead stain contains no bacteria or dead bacteria only). Numbers refer to CFU/mL.

Treatment of five day old biofilms for 21 days

After 21 days, 10,000xMIC inhibited bacteria (Fig 9.3.2.3) and also eradicated them as evident by culture-negativity on resuscitation (Table 9.3.2.2) and absence of live bacteria on the LiveDead stain (Fig 9.3.2.4).

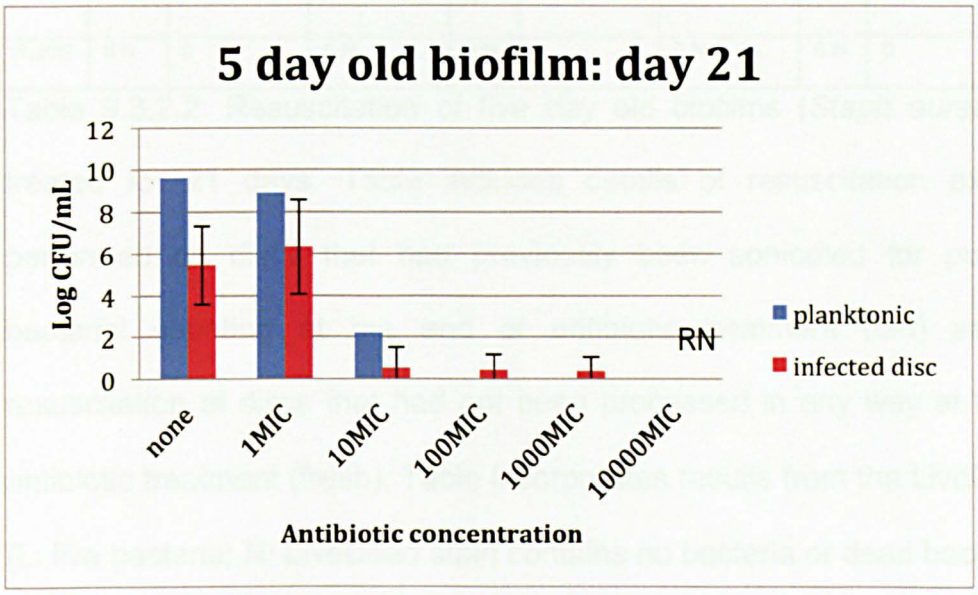


Fig 9.3.2.3: Treatment of five day old biofilms (*S aureus* F2387) with antibiotics for 21 days. RN: resuscitation culture negative. Clean control discs contained no bacteria. Error bars indicate standard deviation.

xMIC	Disc1 CFU / mL			Disc2 CFU / mL			Disc3 CFU / mL		
	In abx	Resuscitated (old)	Resuscitated (fresh)	In abx	Resuscitated (old)	Resuscitated (fresh)	In abx	Resuscitated (old)	Resuscitated (fresh)
10	45N	260000	220000L	0 N	1100000	170000L	0 N	65000	60000L
100	0 N	0	90000L	20 N	170000	160000L	0 N	0	170000L
1,000	0 N	0	0 N	15 N	1920	0 N	0 N	0	1440L
10,000	0 N	0	0 N	0 N	0	0 N	0 N	0	0 N

Table 9.3.2.2: Resuscitation of five day old biofilms (*Staph aureus* F2387) treated for 21 days. Table includes details of resuscitation experiments performed on discs that had previously been sonicated for purposes of bacterial counting at the end of antibiotic treatment (old) as well as resuscitation of discs that had not been processed in any way at the end of antibiotic treatment (fresh). Table incorporates results from the LiveDead stain (L: live bacteria; N: LiveDead stain contains no bacteria or dead bacteria only). Numbers refer to CFU/mL.

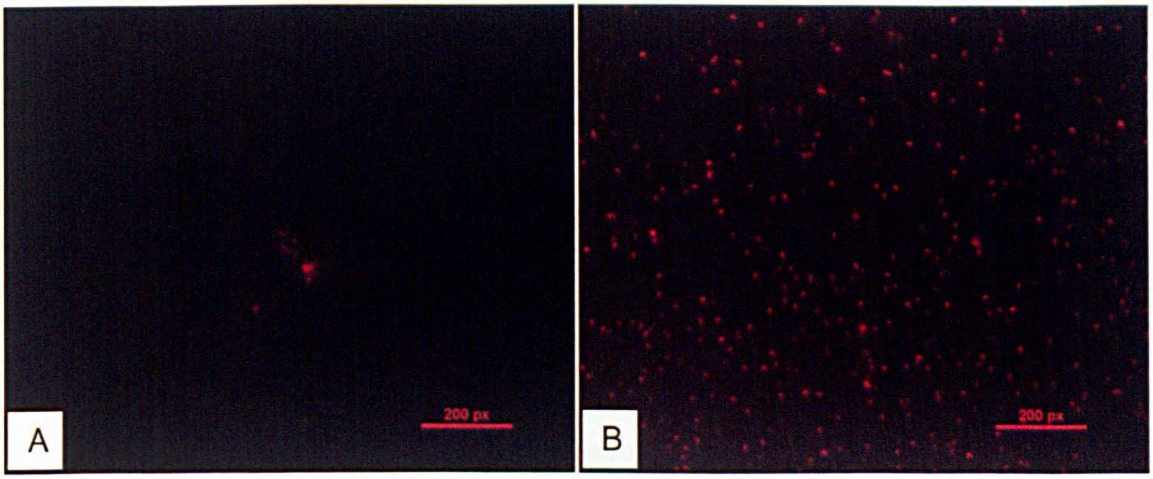


Fig 9.3.2.4: LiveDead stain of five day old (*S aureus* F2387) biofilms treated for 21 days. A: Dead bacteria groups from 1,000xMIC. B: dead individual bacteria from 1,000xMIC

Because those samples that grew bacteria may be culture positive due to emergence of resistance, MIC testing was therefore performed on the positive samples to look for resistance. The rifampicin MIC of the inoculum was 0.003, and the positive samples tested ranged from 0.003 to 0.006, all within the 0.06 µg/mL limit set by BSAC (BSAC 2010). The clindamycin MIC of the inoculum was 0.094, and the samples tested ranged from 0.032 to 0.064, all within the 0.5 µg/mL limit recommended by BSAC (BSAC 2010).

9.3.3 Eradication of five day old biofilms (*S aureus* F2315)

Another strain of *S aureus* (F2315) was also analysed. Treatment of five day old biofilms for one day again failed to eradicate biofilms even at 10,000MIC (Fig 9.3.3.1), and this was also the case in treatment for seven days (Fig 9.3.3.2).

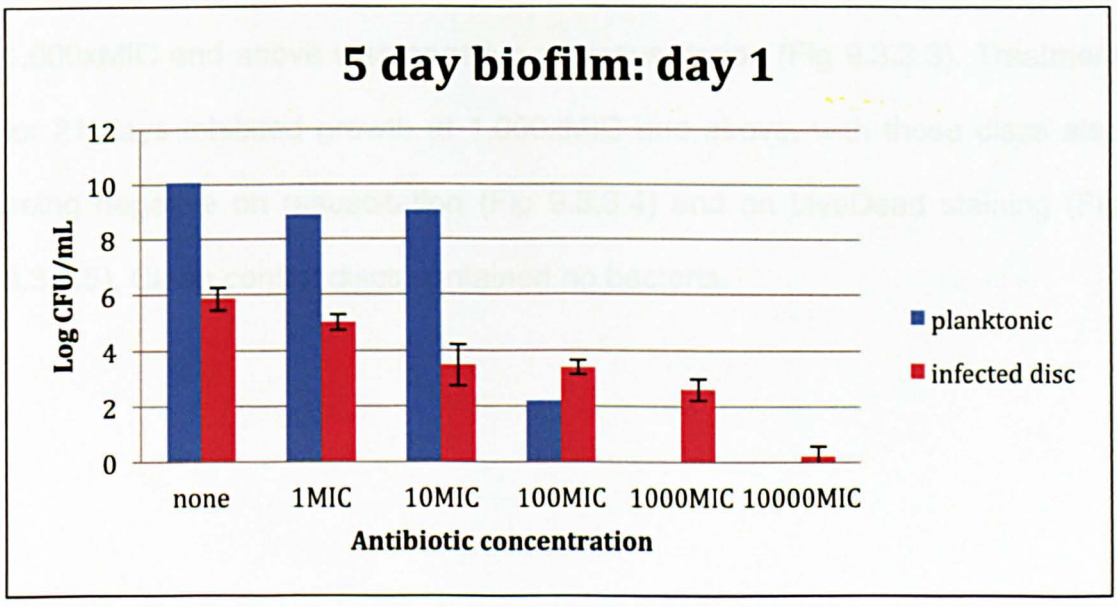


Fig 9.3.3.1: Treatment of five day old biofilms (*S aureus* F2315) with antibiotics for one day. Clean control discs contained no bacteria. Error bars indicate standard deviation.

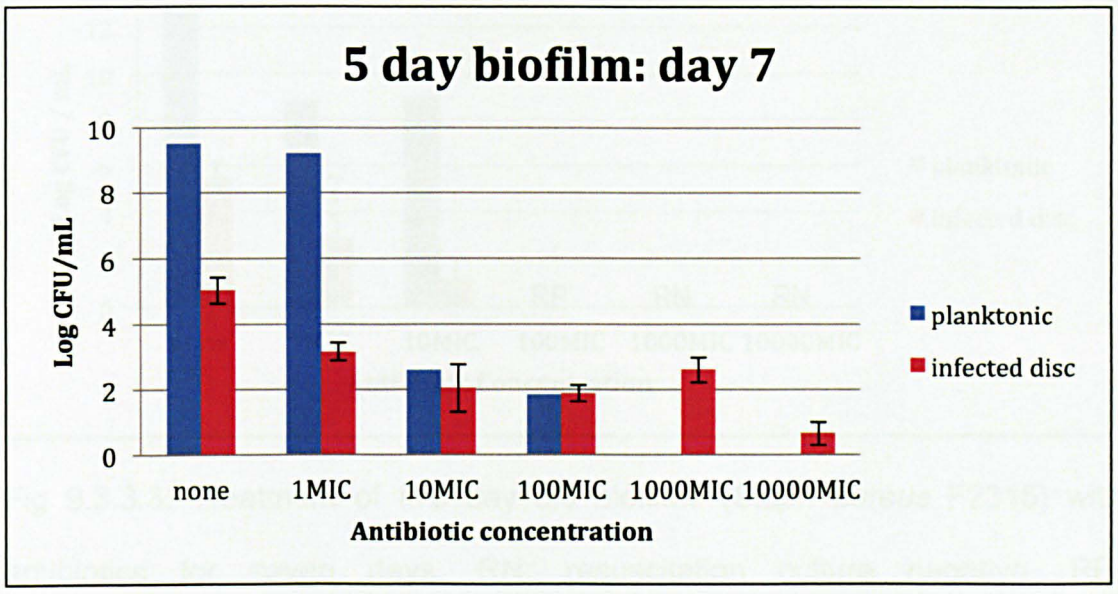


Fig 9.3.3.2: Treatment of five day old biofilms (*S aureus* F2315) with antibiotics for seven days. Error bars indicate standard deviation.

Treatment for 14 days inhibited growth at 100xMIC and above, but only 1,000xMIC and above was negative on resuscitation (Fig 9.3.3.3). Treatment for 21 days inhibited growth at 1,000xMIC and above, with those discs also being negative on resuscitation (Fig 9.3.3.4) and on LiveDead staining (Fig 9.3.3.5). Clean control discs contained no bacteria.

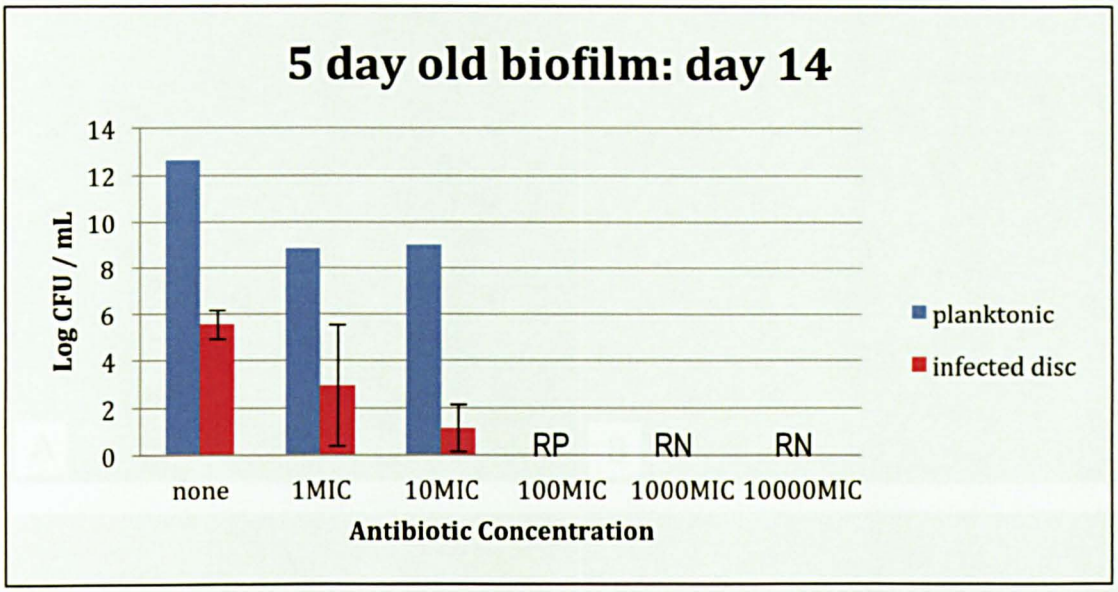


Fig 9.3.3.3: Treatment of five day old biofilms (*Staph aureus* F2315) with antibiotics for seven days. RN: resuscitation culture negative. RP: resuscitation culture positive. Clean control discs contained no bacteria. Error bars indicate standard deviation.

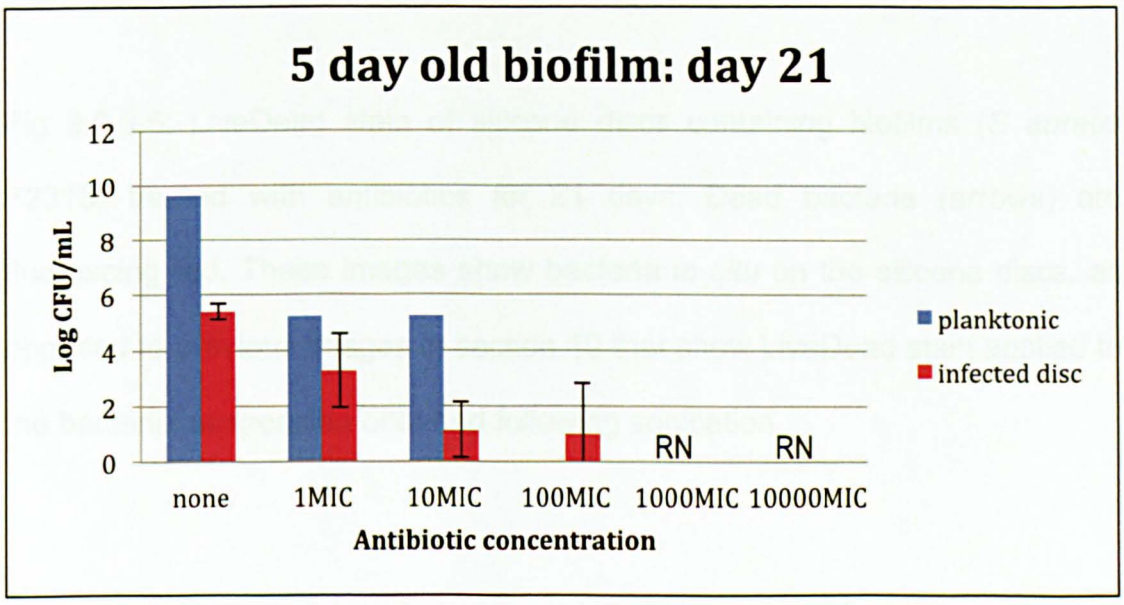


Fig 9.3.3.2: Treatment of five day old biofilms (*Staph aureus* F2315) with antibiotics for seven days. RN: resuscitation culture negative. RP. Clean control discs contained no bacteria. Error bars indicate standard deviation.

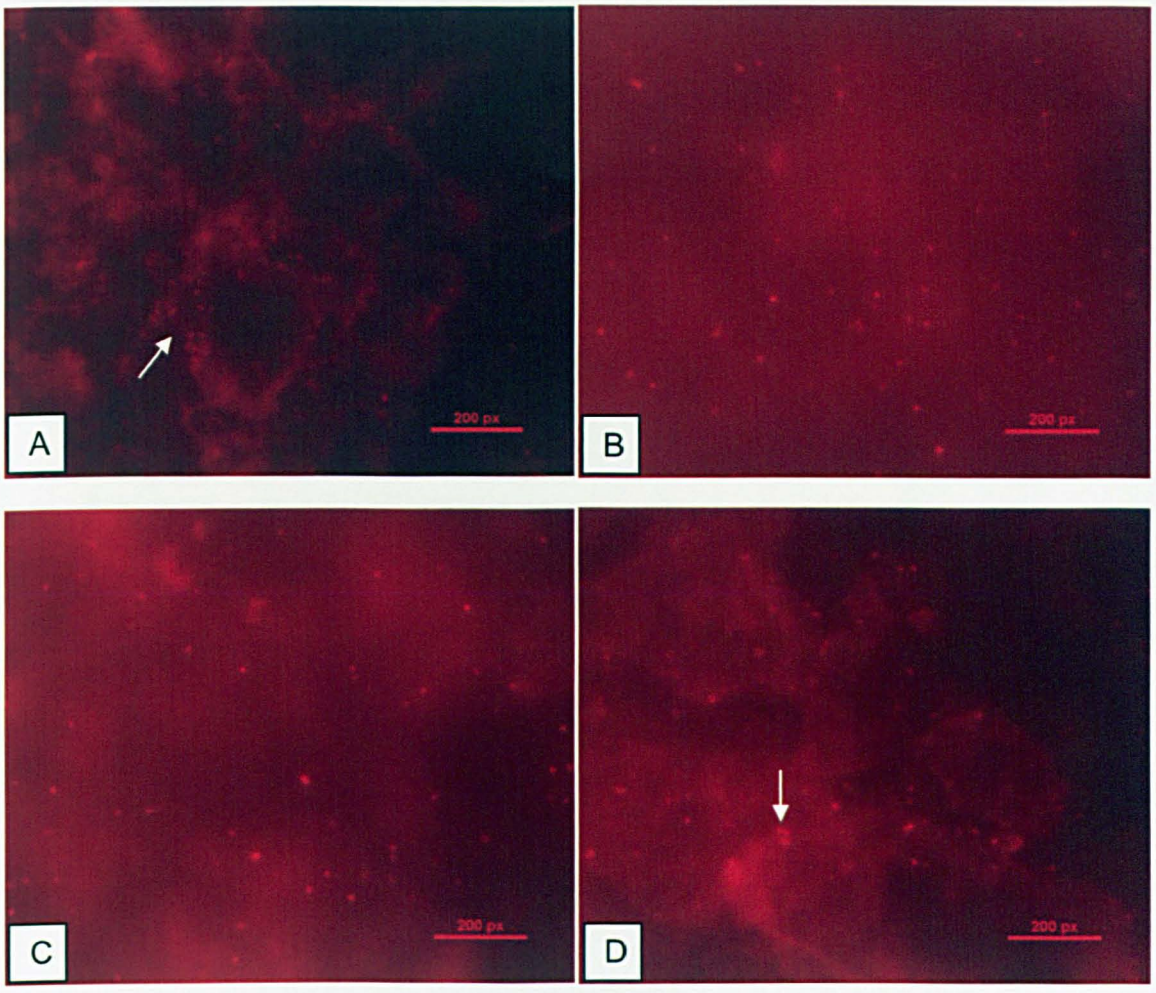


Fig 9.3.3.5: LiveDead stain of silicone discs containing biofilms (*S aureus* F2315) treated with antibiotics for 21 days. Dead bacteria (arrows) are fluorescing red. These images show bacteria *in situ* on the silicone discs, as opposed to previous images in section 10 that show LiveDead stain applied to the bacterial suspension obtained following sonication.

9.3.4 Eradication of 14 day old biofilms

Biofilms that were 14 days old were exposed to antibiotics for 14 and 21 days. Both treatment durations inhibited and eradicated biofilms at 1,000 and 10,000xMIC (Fig 9.3.4.1 and 9.3.4.2)

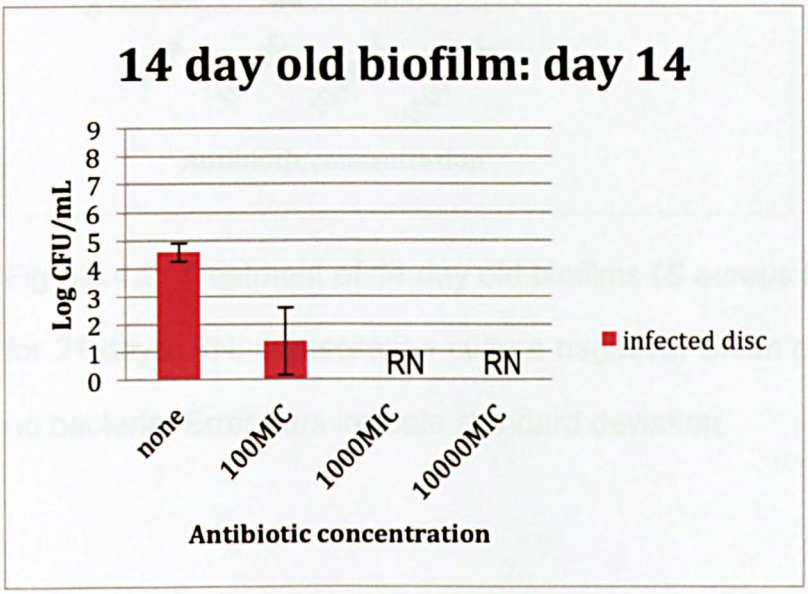


Fig 9.3.4.1: Treatment of 14 day old biofilms (*S aureus* F2387) with antibiotics for 14 days. RN: resuscitation culture negative. Clean control discs contained no bacteria. Error bars indicate standard deviation.

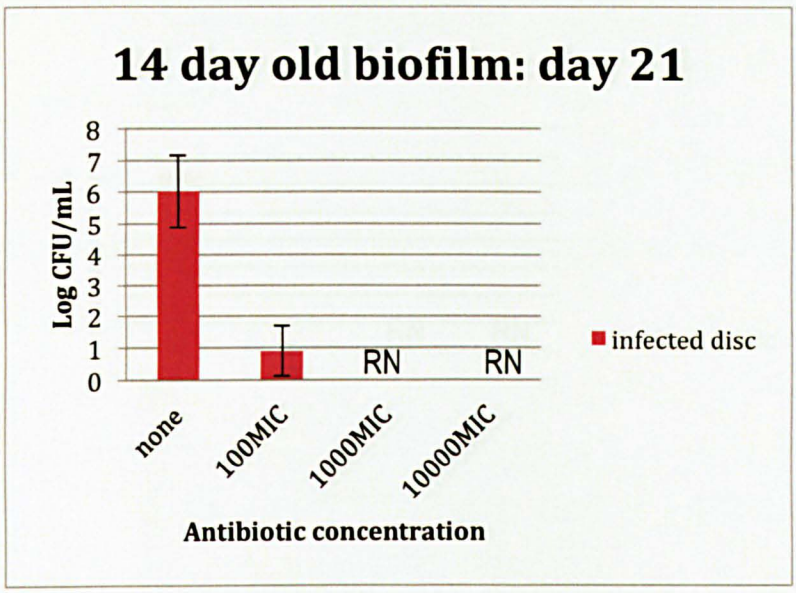


Fig 9.3.4.2: Treatment of 14 day old biofilms (*S aureus* F2387) with antibiotics for 21 days. RN: resuscitation culture negative. Clean control discs contained no bacteria. Error bars indicate standard deviation.

9.3.5 Eradication of 21 day old biofilms

Biofilms that were 21 days old were exposed to antibiotics for 14 and 21 days. Both treatment durations inhibited and eradicated biofilms at 1,000 and 10,000xMIC (Fig 9.3.5.1 and 9.3.5.2).

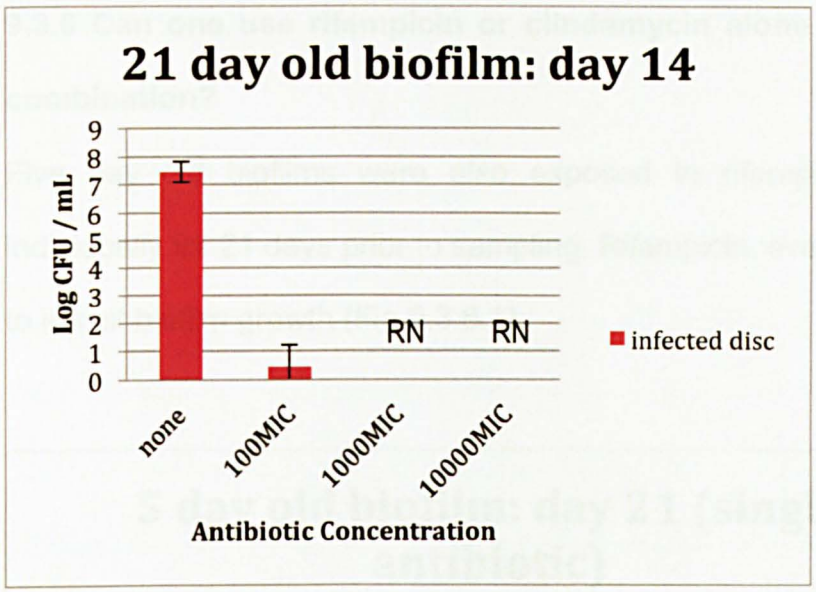


Fig 9.3.5.1: Treatment of 21 day old biofilms (*S aureus* F2387) with antibiotics for 14 days. RN: resuscitation culture negative. Clean control discs contained no bacteria. Error bars indicate standard deviation.

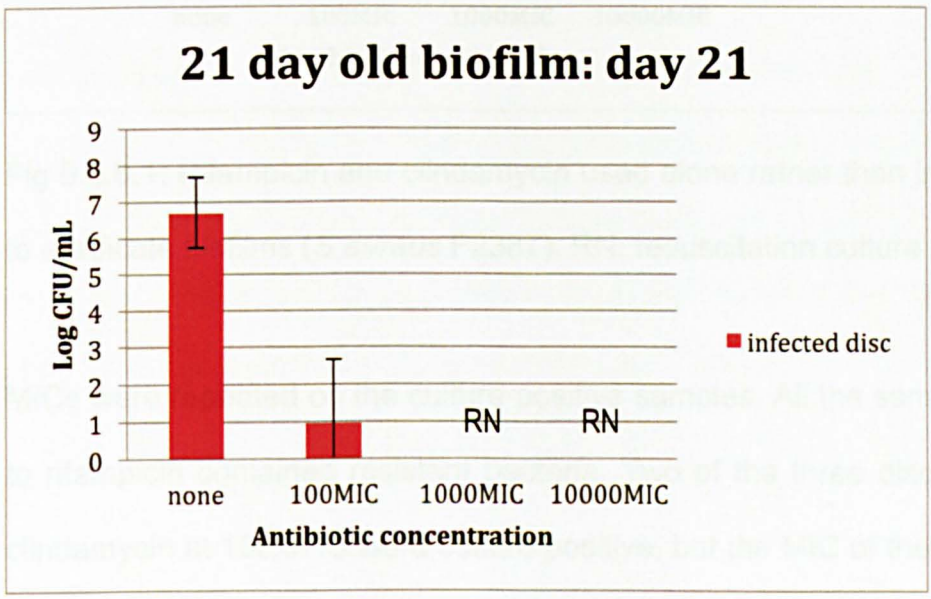


Fig 9.3.5.1: Treatment of 21 day old biofilms (*S aureus* F2387) with antibiotics for 21 days. RN: resuscitation culture negative. Clean control discs contained no bacteria. Error bars indicate standard deviation.

9.3.6 Can one use rifampicin or clindamycin alone rather than both in combination?

Five day old biofilms were also exposed to rifampicin and clindamycin individually for 21 days prior to sampling. Rifampicin, even at 10,000MIC failed to inhibit biofilm growth (Fig 9.3.6.1).

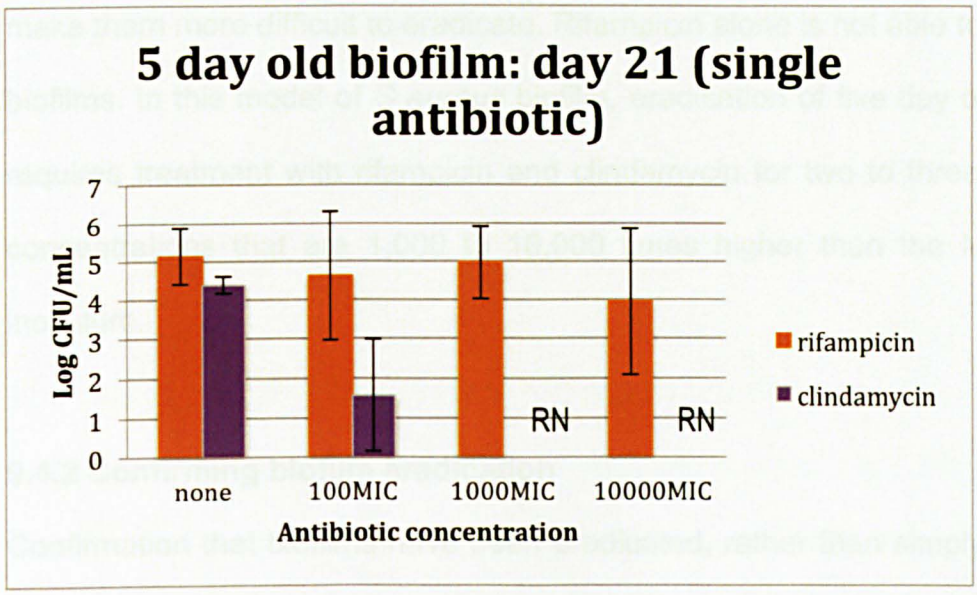


Fig 9.3.6.1: Rifampicin and clindamycin used alone rather than in combination to eradicate biofilms (*S aureus* F2387). RN: resuscitation culture negative.

MICs were repeated on the culture positive samples. All the samples exposed to rifampicin contained resistant bacteria. Two of the three discs exposed to clindamycin at 100xMIC were culture positive, but the MIC of their bacteria did not differ significantly from the inoculum: both MICs were 0.064, whereas the MIC of the inoculum was 0.047.

9.4 DISCUSSION

9.4.1 Summary of findings

Eradication of biofilms using the *in vitro* model could be achieved providing high antibiotic doses are used. One day old biofilms are easier to eradicate than five day old biofilms, but increasing biofilm age to 14 or 21 days does not make them more difficult to eradicate. Rifampicin alone is not able to eradicate biofilms. In this model of *S aureus* biofilm, eradication of five day old biofilms requires treatment with rifampicin and clindamycin for two to three weeks at concentrations that are 1,000 to 10,000 times higher than the MIC of the inoculum.

9.4.2 Confirming biofilm eradication

Confirmation that biofilms have been eradicated, rather than simply inhibited, is important. This is analogous to the difference between minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) when studying planktonic bacteria. In the case of biofilms, their tendency to contain slow growing bacteria or even bacteria that are viable but not culturable means that confirmation that eradication has been achieved is even more important.

This led us to use a 5 day period of "resuscitation", where biofilm bacteria are given optimum growing conditions so that any bacteria that are still alive will start growing and therefore will be culturable (Richards, et al. 2011). This duration was chosen because *S aureus* bacteria are fast growing, and a five

day period would be expected to produce a significant amount of live bacteria that will be detected on culture. Further, the methodology of disrupting biofilm matrix to render bacteria planktonic prior to analysis also means that any live bacteria are likely to be detected, and thus any culture negative samples are genuinely negative.

The LiveDead stain was also used, as this would identify as "live" even those bacteria that are not culturable. The good correlation between LiveDead staining and culture, discussed in the previous chapter, suggests that any treatment regimes that have no culturable bacteria are genuinely negative.

9.4.3 Biofilm eradication and the influence of biofilm age

Biofilms are characterised by their reduced susceptibility to antibiotics, typically requiring from 10 to 1000 times higher antibiotic levels than those required to inhibit planktonic bacteria (Donlan and Costerton 2002) (Vorachit, et al. 1993) (Nickel, et al. 1985) (Ceri, et al. 1999) (Olson, et al. 2002). A variety of reasons are responsible for this as discussed in the introduction and summarised in Fig 9.4.3.1.

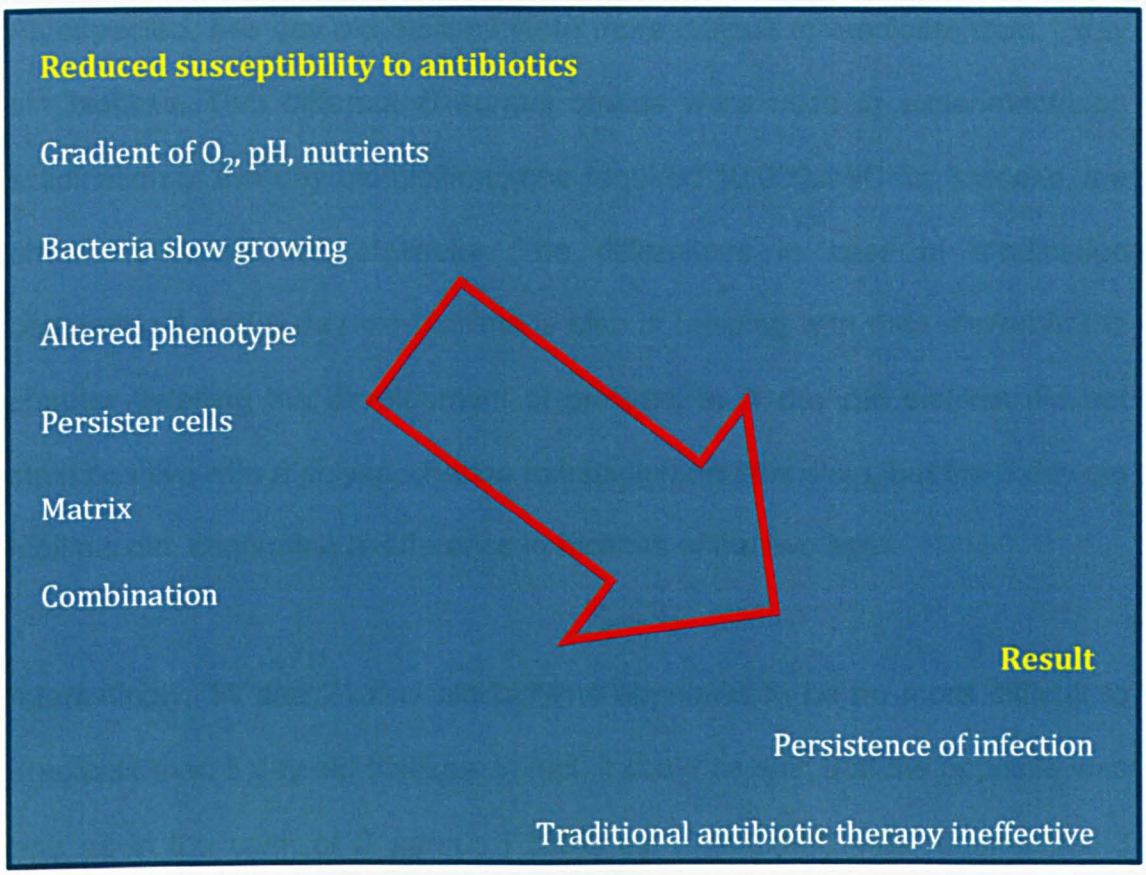


Fig 9.4.3.1: Biofilms are recalcitrant to antibiotics for a variety of reasons

This study shows that one day old biofilms could be eradicated by treating with antibiotics at 100xMIC for 3 weeks, or at 10,000xMIC for five days or longer. One may intuitively suspect such a relationship between dose and duration i.e. higher dose requires shorter exposure time and lower dose requires longer treatment time, and this may also be explained by the complex pharmacodynamics of rifampicin and clindamycin; both can be bacteriostatic or bactericidal depending on dose and exposure time, and exhibit both time dependent and concentration dependent killing (Gumbo, et al. 2007) (Bakker-Woudenberg, et al. 2005) (Klepser, et al. 1996) (Aldridge and Stratton 1991). Thus both the time as well as antibiotic level are important.

As expected, five day old biofilms were more difficult to eradicate than 1 day old biofilms. Two different *S aureus* strains were used in experiments on eradication of five day old biofilms, one required 10,000xMIC for 3 weeks, the other 1,000xMIC for 2 weeks. The differences in ease of eradication comparing 1 and 5 day old biofilms is also in keeping with data shown in the chapter detailing the development of biofilms, as 1 day old biofilms did not stain positive with a polysaccharide extracellular matrix stain, but the 5 day old biofilms did, confirming a difference in biofilms of the two ages.

Interestingly, 14 and 21 day old biofilms appeared to be no more difficult to eradicate than 5 day old biofilms; in fact, it could be said that the opposite was true as in the case of *S aureus* F2387, as 10,000xMIC was required for 3 weeks to eradicate 5 day old biofilms, but only 1,000xMIC for 14 days could eradicate 14 and 21 day old biofilms. One might speculate that differences in methodology may account for this unexpected difference, but methodology was standardised, and the inoculum kept the same. It is possible that older biofilms exhaust the nutrient supply and may be easier to eradicate, but on the other hand lack of nutrients may actually make them more likely to adopt a slow growing state and shut down non-essential functions and thus become more recalcitrant. The observation that no-antibiotic control biofilms did not die suggests that nutrients were sufficient for biofilm survival.

It is also interesting that the 1,000xMIC concentration for 2 weeks eradicates the 5 day old biofilms formed by *S aureus* F2315 and also 14 and 21 day old biofilms formed by F2387, but 5 day old biofilms of *S aureus* F2387 require

higher concentration for longer. One might speculate that this appears to be an anomaly, and the reasons for it are not immediately apparent. It is important to note actual bacterial numbers, rather than just presence of eradication; in the case of 5 day old F2387 treated for 2 weeks at 10,000xMIC only one of the triplicate biofilm discs was in fact positive on culture with just 15 CFU/mL, and all three discs were in fact culture negative on resuscitation, suggesting that whilst 10,000xMIC for 14 days might be considered as failed eradication the actual number of surviving bacteria is extremely small.

The influence of biofilm age is an important consideration when planning OME treatment, as the biofilms are likely to be months old by the time treatment is instituted (see also chapter 8). Therefore, the observation that 2 or 3 week old biofilms appear not to be more difficult to eradicate is welcome, although we have not examined biofilms that are several months old *in vitro*. One may expect older biofilms to be more difficult to eradicate (Exterkate, Crielaard and Ten Cate 2010) (R. Donlan 2001), but this has not been a universal finding with other studies not finding biofilm age to be a significant factor influencing eradication (Wong, et al. 2010) (Wilson, Patel and Fletcher 1996).

The antibiotic concentrations in this biofilm model were kept constant. While a small amount of deterioration in antibiotic potency over the three weeks of each experiment may occur, it is unlikely that a significant drop in antibiotic activity will result. Therefore, whilst one can comment on biofilm eradication using a pre-determined constant concentration, the experiments described here do not provide any information on the possibility of varying the antibiotic

levels during a course of treatment aimed at biofilm eradication. A literature search was unable to find many papers that examined variation in antibiotic level over time in biofilm treatment. One paper examining modified release of levofloxacin against *E coli* biofilms suggested that a high initial level is crucial, because the modified release formulation that gave a very strong burst release and subsequent low levels was more effective against biofilms than an alternative formulation that released antibiotic more gradually even though the latter formulation gave higher antibiotic levels after the first day (Cheow, Chang and Hadinoto 2010). It is possible that biofilm eradication requires the very high concentration of antibiotic only for an initial critical time period (say a few days), with a lower level being sufficient treatment for the remainder of the two to three week period. This is of direct relevance when discussing a modified release antibiotic formulation against biofilms in the next section, because many modified release devices do release a higher level of drug at the start.

Looking at those results overall, it appears that a strategy utilising antibiotics at 1,000 to 10,000xMIC for 2-3 weeks is likely to be effective at eradicating biofilms.

9.4.4 Choice of antibiotics

Rifampicin and clindamycin were chosen as they both work against *S aureus* that was a common OME organism in our previous work and the focus of the *in vitro* experiments outlined here. Between them they also cover the other pathogens that published literature suggests are common in OME

(*Streptococcus pneumoniae, Moraxella catarrhalis, Haemophilus influenzae*) (Thornsberry, et al. 1999).

Rifampicin is effective against most Gram positive cocci including Staphylococci and Streptococci, and is also effective against *Haemophilus influenzae*. In the case of clindamycin, most aerobic Gram negative bacteria (including *Haemophilus, Moraxella*) are resistant, but aerobic Gram positive cocci (including Staphylococci and Streptococci) and anaerobic Gram negative rods are sensitive. Both rifampicin and clindamycin can be either bacteriostatic or bactericidal depending on dose and exposure time, and exhibit both time dependent or concentration dependent killing (Gumbo, et al. 2007) (Bakker-Woudenberg, et al. 2005) (Klepser, et al. 1996) (Aldridge and Stratton 1991).

Both rifampicin and clindamycin also have particular advantages in the treatment of biofilms. Although not universally accepted, it is thought by some that rifampicin and clindamycin may be able to penetrate the biofilm matrix better than other antibiotics (Souli and Giamarellou 1998), and may have better anti-biofilm activity (Lee, et al. 2006). Much information exists on using rifampicin in combination with other antibiotics, showing impressive results in the management of biofilm infections with a strong synergistic bactericidal activity (Barriere 2006) (Gomes, et al. 2012) (John, et al. 2009), suggesting that rifampicin may have substantial anti-biofilm properties over and above its activity against planktonic bacteria. The rifampicin /clindamycin combination has also ben used effectively in osteoarticular infections (Czekaj, et al. 2011).

9.4.5 The use of two antibiotics to minimise emergence of resistance

Antibiotic resistance is a major health care concern (D'Agata, et al. 2008) (Olofsson and Cars 2007). Development of resistance can be influenced both by bacteria specific factors and by antibiotic pressures. Many bacteria undergo rapid division with a risk of mutation that may confer antibiotic resistance, but some bacteria in a population can have a mutation rate that is 10 to 10,000 times higher than the mutation rate in the wild type (Hall and Henderson-Begg 2006). Although bacteria with genetic resistance to antibiotics may have reduced biological fitness and therefore in a mixed population the sensitive bacteria are likely to predominate, when antibiotics are present the resistant bacteria in a mixed population are selected over the sensitive bacteria and then become the predominant type. How antibiotics are administered also affects emergence of resistance, with low doses favouring resistance and high antibiotic levels favouring eradication (Olofsson and Cars 2007).

An important consideration in the OME work presented here was the use of two antibiotics to minimise emergence of resistance. This works on the principle that, whilst bacteria may acquire resistance to one antibiotic, they will remain sensitive to the other antibiotic, and the simultaneous acquisition of resistance to two different antibiotics would be a rare event (D'Agata, et al. 2008) (Olofsson and Cars 2007) (Bonhoeffer, Lipsitch and Levin 1997) (Lipsitch and Levin 1997). This is particularly relevant in the case of rifampicin, where only a single mutation in the rpoB gene (Valvatne, et al. 2009) leads to

development of resistance. Indeed, our experiments on using antibiotics singly rather than in combination confirms the development of resistance to rifampicin and thus failure of biofilm eradication.

Although rifampicin and clindamycin both cover *Staph aureus* used in these experiments, if other common OME bacteria were tried then these two antibiotics may not provide double coverage for each species. The use of rifampicin and clindamycin was appropriate in these experiments on *S aureus*, but future work should consider other bacteria and thus adding in another antibiotic to ensure that each bacterial species is covered by at least two antibiotics to minimise resistance. An appropriate additional antibiotic for future study would be amoxicillin.

9.4.6 Achieving high antibiotic concentrations in the middle ear

The results indicate that treatment with antibiotic levels at 1,000x to 10,000xMIC are likely to be required to eradicate biofilms. In the case of 5 day old *S aureus* biofilms grown on silicone discs this means a rifampicin level of 6 - 60 mg/L and clindamycin level of 100 – 1000 mg/L. Although translating this into clinical treatment of OME is a long leap, how could such levels be achieved in the middle ear?

After systemic administration, rifampicin typically reaches plasma concentrations of up to 32 mg/L (Inchem 2011) (Phillips 1971), which would be broadly satisfactory based on the *in vitro* data presented here. However, as already stated rifampicin cannot be used alone. Clindamycin administered

systemically (orally) achieves levels of 6 mg/L (Phillips 1971), way below the required 1,000xMIC or even 100xMIC. Thus, systemically administered rifampicin and clindamycin combination would not be expected to eradicate biofilms in the middle ear based on (long shot) interpolation from the *in vitro* model.

Another issue to consider is the transfer of antibiotics from plasma to the middle ear cavity and mucus, as antibiotic levels in the middle ear may not be as high as those in blood. A literature search did not identify any studies examining rifampicin and clindamycin levels in the middle ear. However, a study of erythromycin showed that although levels in the middle ear effusion do not rise as fast as plasma levels after systemic administration, the levels in middle ear do reach those seen in plasma and stay higher in the middle ear for longer (Sundberg, et al. 1979). Levels of systemically administered ofloxacin were also similar in plasma and in middle ear effusion, but only reached levels 4 times higher than the MIC of *S aureus* (Thorn 1987). Another study of several different antibiotics found levels in the middle ear to be several fold greater than MICs of *Strep pneumoniae*, but only trimethoprim and cefaclor levels exceeded the MIC of *Haemophilus influenzae* (Nelson, et al. 1981).

The results of the *in vitro* experiments on biofilms eradication suggest that systemic antibiotic administration would be unlikely to achieve sufficiently high antibiotic levels in the middle ear to eradicate biofilms. Therefore, a local

delivery strategy is of interest, and became the focus of our further research as detailed in the next thesis chapter.

Any strategy that delivers high dose antibiotics (and a delivery vehicle) to the middle ear must be safe, both to the middle ear and the inner ear that communicates with the middle ear. In particular, the inner ear is sensitive to a variety of different agents, with potential for disastrous permanent hearing loss and balance problems in cases of inner ear damage. The delivery mechanism must also not damage the middle ear (see also Chapter 10.4.5), otherwise inflammation due to bacteria is simply replaced by inflammation due to the drug delivery vehicle. Although one can chose antibiotics thought to be safe, further research (for example *in vivo* oto-toxicity studies looking at middle and inner ear, hearing and balance) would be required prior to clinical use. In addition, initial clinical studies should themselves incorporate safety studies before an efficacy trial is undertaken, and even after widespread clinical use mechanisms to report concerns should be in place.

The data on antibiotic levels in plasma and the middle ear also provide useful information on interaction of antibiotics with mucus. As a planned future OME strategy may include delivery of antibiotics direct to the middle ear, the interaction of antibiotics and mucus is important. To be effective, the antibiotic would have to penetrate the mucus to get to the biofilms. The observation that antibiotic levels in the middle ear effusion are comparable to those in plasma suggests that the mucus does not interfere with antibiotic diffusion. However, the investigation of antibiotic diffusion through mucus should form an

important part of future work investigating direct delivery of antibiotics to the middle ear to treat OME. A suitable experiment would include placing the antibiotic pellet onto a bed of mucus (held in a mould but with open base), and placing the mucus on an inoculated agar plate and measuring the zone of inhibited bacterial growth (Fig 9.4.5.1).

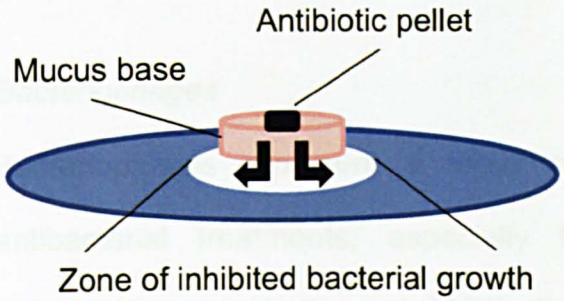


Fig 9.4.6.1: Suggested model for investigating diffusion of antibiotic through mucus

9.4.7 Other anti-biofilm strategies

Given that eradication of biofilms with antibiotics is difficult, requiring high antibiotic levels, much research has focused on treatment of biofilms with other agents (Donlan and Costerton 2002) (Aslam 2008) (Sihorkar and Vyas 2001) (Foreman, Jervis-Bardy and Wormwald 2011). A number of promising strategies are currently being investigated, and future work may establish which genes or proteins are involved in attachment, biofilm formation, and phenotype change, and any of these may also represent a potential therapeutic target (Lewis 2001).

Broadly speaking, anti-biofilm strategies (Foreman, Jervis-Bardy and Wormwald 2011) can be classified as follows into those that:

- Affect individual bacteria i.e. traditional antibiotics
- Physically disrupt surface attachments
- Free bacteria from the biofilm, either by degrading the matrix or promoting a phenotypic shift towards planktonic state

Bacteriophages

Bacteriophages represent a focus of much research looking at novel antibacterial treatments, especially for resistant bacteria (Vinodkumar, Kalsurmath and Neelagund 2008). Bacteriophages are viruses that infect bacteria. They are themselves effective against biofilms (Soni and Nannapaneni 2010), and also potentiate the effects of antibiotics (Verma, Harjai and Chhibber 2010). Importantly, they have investigated in a clinical trial that found bacteriophages to be effective and safe in patients with antibiotic-resistant *Pseudomonas aeruginosa* causing ear infections (Wright, et al. 2009).

Quorum sensing

Quorum sensing may be an interesting future strategy. It refers to the way bacteria use signalling molecules to sense other bacteria (either of the same or of a different species) and alter their behaviour as a result (Miller and Bassler 2001). The bacteria produce and release chemical signal molecules that regulate gene expression and thus affect population density and a variety of physiologic processes. This allows bacteria to take advantage of knowledge

of other bacteria in the vicinity, and from an evolutionary point of view is thought to a precursor to multi-cellular existence. Quorum sensing is thought to be important in biofilm formation, and inhibition of quorum sensing has been shown to affect biofilm formation (Taganna, et al. 2011) (Khalilzadeh, et al. 2010) and this represent a possible future therapeutic target (Williams 2007) (Bassler and Losick 2006) (Diggle, Crusz and Camara 2007). However, some research has suggested that quorum sensing inhibition may promote the growth of more virulent mutants (Kohler, et al. 2010), and that some quorum sensing pathways may in fact repress biofilm formation (Xu, et al. 2006); therefore, further investigation is required before any clinical use.

Matrix disruption

Disruption of the matrix is another possible anti-biofilm strategy. In addition to agents that degrade extracellular matrix (Johansen, Falholt and Gram 1997), this could also be achieved with physical methods such as ultrasound (Huang, et al. 1996) (Ensing, et al. 2006). Physical disruption may also be the reason for the effectiveness of nasal douching in chronic rhinosinusitis, another infection where biofilms are thought to be important (Fokkens, et al. 2007). Disruption of the matrix will affect the biofilm itself, but may release bacteria into the planktonic state thus rendering them susceptible to traditional antibiotic therapy or the immune system (Boles and Horswill 2011). In this context, the use of N acetyl cysteine (NAC) to disrupt the biofilm matrix may be a useful strategy (del Prado, et al. 2011) (El-Feky, et al. 2009) (Venkatesh, et al. 2009) (Al-Zahid, et al. 2011), as may the use of DNase to disrupt

extracellular DNA that is an adhesive component of biofilms (Kaplan, et al. 2012).

NAC, a mucolytic already in clinical use in respiratory conditions, is of particular interest in biofilm treatment and OME. NAC has been shown to enhance biofilm eradication when combined with antibiotics (del Prado, et al. 2011) (El-Feky, et al. 2009) (Venkatesh, et al. 2009) (Al-Zahid, et al. 2011), but it also has anti-biofilm properties on its own (Naves, et al. 2010) (Zhao and Liu 2010) (Olofsson, Hermansson and Elwing 2003). *In vitro* studies of NAC's mucolytic properties have also shown that NAC was able to reduce the viscosity of middle ear effusions (Fitzgerald, et al. 1988); thus, disrupting mucin may also remove a potential site of biofilm attachment.

Further, and importantly, the anti biofilm activity of NAC has also been translated into clinical benefits. One study of *Helicobacter pylori* eradication found that administering NAC to patients prior to antibiotics increased the success rate (Cammarota, et al. 2010). In the treatment of OME, a clinical trial of topical NAC administration at time of VT insertion and for a week post operatively found that the incidence of ear discharge was reduced, VTs stayed in place for longer before extrusion, and the need for revision surgery was reduced (Ovesen, et al. 2000). However, although animal studies suggest a reduction in scarring of the tympanic membrane (Ozcan, et al. 2002), they also found that NAC inhibited the healing of tympanic membrane with increased risk of perforation (Sanli, et al. 2007).

NAC is already in clinical use in the treatment of paracetamol overdose (it replenishes the body's stores of antioxidant glutathione) and in the management of respiratory problems such as chronic bronchitis, although it is not clear whether the benefits arise as a result of NAC's mucolytic, anti-inflammatory or anti-oxidant properties (Stey, et al. 2000).

Advantages of antibiotics in biofilm eradication

Given the concerns about antibiotic resistance and the development of several new antibacterial strategies outlined above, what is the advantage of using antibiotics? The focus of biofilm eradication in this study was the use of antibiotics because they have been in widespread clinical use already, much is known about them, and their use involves relative simple adaptation of existing knowledge. The downside of the newer techniques under investigation is that, although they may well be useful in the future, at present they may not be in clinical use and much remains unknown about them. Therefore, if one wishes to improve OME treatment as soon as possible then the adoption of tried and tested techniques is likely to yield the best results in the shortest time.

Nevertheless, NAC has already been shown to have clinical benefit in OME, with an increasing amount of recent literature appreciating its anti biofilm properties, either alone or in conjunction with antibiotics (and in addition to its anti-oxidant and anti-inflammatory properties). Therefore, the use of NAC in OME also warrants further investigation, likely as an adjunct to antibiotic treatment.

9.5 BIOFILM ERADICATION WITH ANTIBIOTICS: CONCLUSION

Using the *in vitro* model of *S aureus* biofilms on silicone discs treated with rifampicin and clindamycin, it can be shown that eradication of biofilms with antibiotics is possible, but requires treatment with high antibiotic levels (typically 1,000 to 10,000 times higher than the level required to inhibit planktonic bacteria) for a period of two to three weeks. The use of two antibiotics to minimise emergence of resistance is also justified.

Thus, a new strategy to eradicate middle ear biofilms in OME using high antibiotic levels for several weeks could be feasible. However, these high levels would be difficult to achieve with systemic treatment and may risk systemic toxicity, so delivery locally to the middle ear, the site of the biofilm, is of interest. The next chapter describes a potential novel approach using modified release antibiotic pellets.

10 BIODEGRADABLE MODIFIED RELEASE ANTIBIOTIC PELLETS

10.1 INTRODUCTION

Having established that biofilms are implicated in the aetiology of OME, and that treatment with antibiotics can eradicate biofilms in an *in vitro* model, we wished to translate this knowledge into improved treatment of OME. Biofilm eradication would require high antibiotic doses, and treatment over several weeks. As systemic administration would be unlikely to achieve high enough levels of both rifampicin and clindamycin in the middle ear, local delivery is the logical solution to achieve this whilst minimising any systemic toxicity. As treatment over several weeks is needed, a modified-release formulation is required.

The current treatment with Ventilation Tube (VT, grommet) insertion involves a general anaesthetic, with the surgeon performing a myringotomy (creating an incision in the ear drum to gain access to the middle ear), aspirating the middle ear effusion, and then inserting the VT into the ear drum. The fact that the myringotomy is already part of clinical care means that access to the middle ear, where the biofilms are, is possible, but only on the occasion of surgery. However, as biofilm eradication requires treatment over several weeks, a modified release formulation would be required. This modified release device can then be inserted into the middle ear at the time of surgery,

and release antibiotics over several weeks. It would have to be biodegradable to avoid need for removal surgery. The desired clinical outcome would be a reduction in the recurrence of OME after VT extrusion, and therefore a reduction in the current high need for repeat VT insertion.

Poly (lactic-co-glycolic acid) (PLGA), which already has a history of safe clinical use (Lu, et al. 2009), was chosen as the base for a modified release formulation. We aimed to produce the antibiotic pellets using PLGA microparticles smaller than 50 μm in diameter. This chapter details the production of PLGA microparticles and the pellets themselves. In addition, the choice of modified release formulation is discussed, including the advantages and disadvantages of the chosen formulation incorporating PLGA mixed with either poloxamer or carboxymethylcellulose.

10.2 MATERIALS AND METHODS

10.2.1 Production of microparticles using the milling method

PLGA (Lakeshore Biomaterials, Birmingham, USA) was placed into a Krups F203 grinder (Krups, Solingen, Germany) together with liquid nitrogen to keep the PLGA cool (PLGA is thermo-sensitive and would be affected by the heat generated by the grinder). After grinding for a few seconds the mix was sieved and particles larger than the desired size re-ground.

10.2.2 Production of microparticles using the emulsion method

The oil/water emulsion method was used to prepare microparticles. 1g of PLGA was dissolved in 6.7 mL of dichloromethane (DCM) (Sigma-Aldrich, Gillingham, UK) in a glass container, and added to 200 mL of 0.3% polyvinyl alcohol (PVA) (Sigma-Aldrich, Gillingham, UK). The mix was homogenised using a Silverson L5M mixer for 2 minutes at 9000RPM (Silverson Machines, Chesham, UK) (Silverson 2010). The emulsion, at room temperature, was then left stirring overnight (16 hours) with a 50 mm glass flea on Variomag Poly magnetic stirrer (Variomag, Daytona Beach, USA) set at 300rpm, to allow DCM to evaporate.

The following day, particles were removed from the PVA emulsion. The PVA / PLGA emulsion was aliquoted into 50 mL containers, and centrifuged at 3000 rpm for 5 minutes (MSE Mistral 1000, London, UK). The supernatant was discarded, and the particles re-suspended in deionised water and centrifuged

again. The step was repeated until the samples had been centrifuged three times in water.

The resulting PLGA suspension was filtered through a 40μm filter to remove any large clumps, frozen in liquid nitrogen and then freeze dried (ModulyoD, Thermo Electron Corporation, Waltham, MA USA) prior to storage at -20°C.

10.2.3 Determining the size of the microparticles

The size of the particles was determined by laser diffraction. In this technique, a suspension of particles is passed through a broadened beam of laser light, which scatters the incident light onto a Fourier lens that focuses the scattered light onto a detector. The particle size is inferred from the collected diffracted light data. The PLGA microparticles were suspended in deionised water to achieve 8-12% obscuration, stirred continuously, and measured with Coulter LS230 particle size analyser (Beckman Coulter, High Wycombe, UK, solid state 780nm laser) using the Garnett optical formula.

10.2.4 Production of antibiotic pellets

The pellets were composed of PLGA (Lakeshore Biomaterials, Birmingham, USA), either 2% high viscosity sodium carboxymethylcellulose (Blanose®, Aqualon / Hercules, Widnes, UK) or 20% Poloxamer Pluronic F127 (Sigma-Aldrich, Gillingham, UK), and antibiotics rifampicin (Sigma-Aldrich, Gillingham, UK) and clindamycin (Sigma-Aldrich, Gillingham, UK).

The pellets were produced by mixing PLGA microparticles with a carrier solution and antibiotics. The carrier solution had been autoclaved, and the mix prepared in a clean environment. The pellet components were sterilised by exposure to ultraviolet (UV) light (UVG-54 Handheld UV lamp 254 nm UV / 6 Watt / 230V, Upland, USA) for 15 minutes prior to mixing by hand. The mix was placed into a PTFE mould (Fig 10.2.4.1) designed to produce pellets 3 mm long and 2.5 mm diameter, with a central 1mm diameter hole (the result of a stainless steel pin). The pellets were sintered at 60°C for a pre-determined period of time, taken out of the mould by removing the top plate, and sterilised again with UV light. The pellets were stored at 4°C and used within a week of preparation.

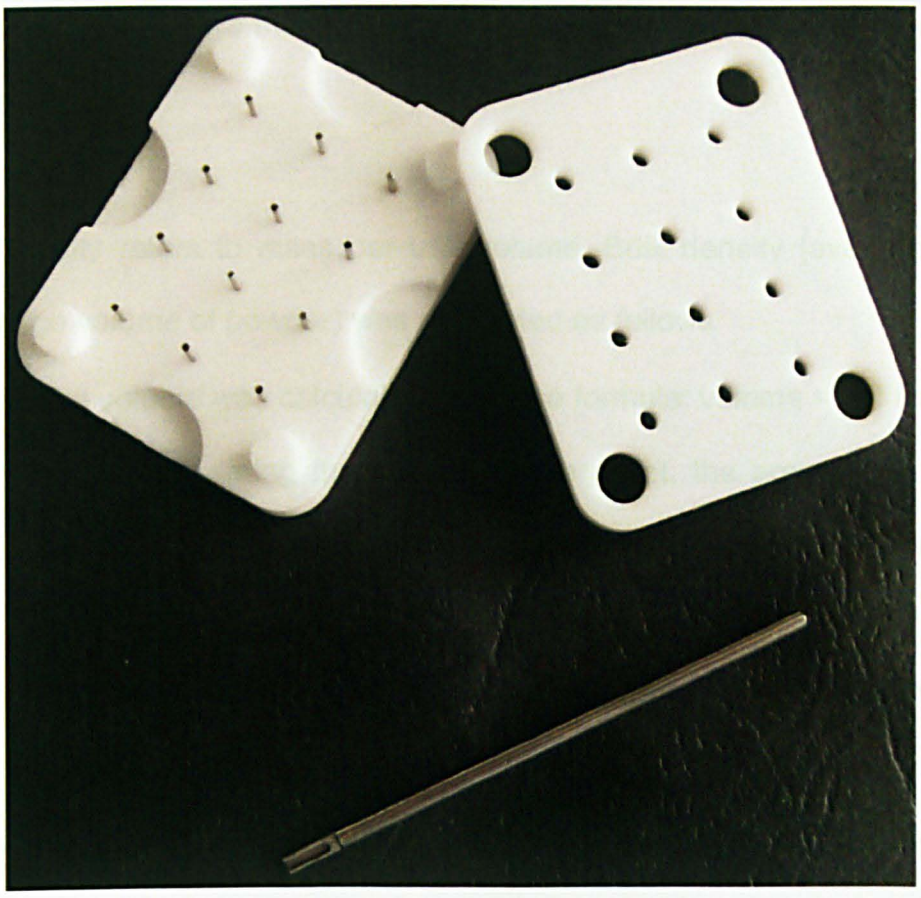


Fig 10.2.4.1: Photograph of the PTFE / stainless steel mould for the antibiotic pellets showing base with stainless steel pins, perforated top plate, and compacting tool.

10.2.5 Scanning electron microscopy

SEM was used to examine microparticles and antibiotic pellets, which were sputter coated with gold for 4 minutes prior to examination with Jeol 6060LV variable pressure SEM (Jeol UK Ltd, Welwyn Garden City UK).

10.2.6 Measuring density and porosity

The pellets were frozen in liquid nitrogen and then freeze-dried (ModulyoD, Thermo Electron Corporation, Waltham, MA USA) overnight. The dimensions

of the pellet were measured using electronic callipers, and they were weighed accurately.

Density refers to mass per unit volume. Bulk density (average density of a large volume of powder) was calculated as follows

- The volume was calculated using the formula: volume = π * radius^2 x length (When calculating the volume of the pellet, the area of the central 1mm diameter hole was subtracted to obtain the final volume of just those areas containing microparticles)
- Bulk density was calculated using the formula: bulk density = weight / volume
- Porosity was calculated using the formula: porosity (%)= (1 – (bulk density / particle density)) * 100
- The particle density of PLGA (density of particles making up a powder) is 1.25 g/cm (Leung, et al. 2008).

10.2.7 Sterility assessment

Inactive pellets containing no antibiotic prepared and sterilised as described in general methods were incubated (in triplicate) in TSB for 5 days, and the TSB then plated on sheep blood agar to establish whether the pellets prepared in this way contained bacteria that would grow on blood agar.

10.3 RESULTS

10.3.1 Production of microparticles

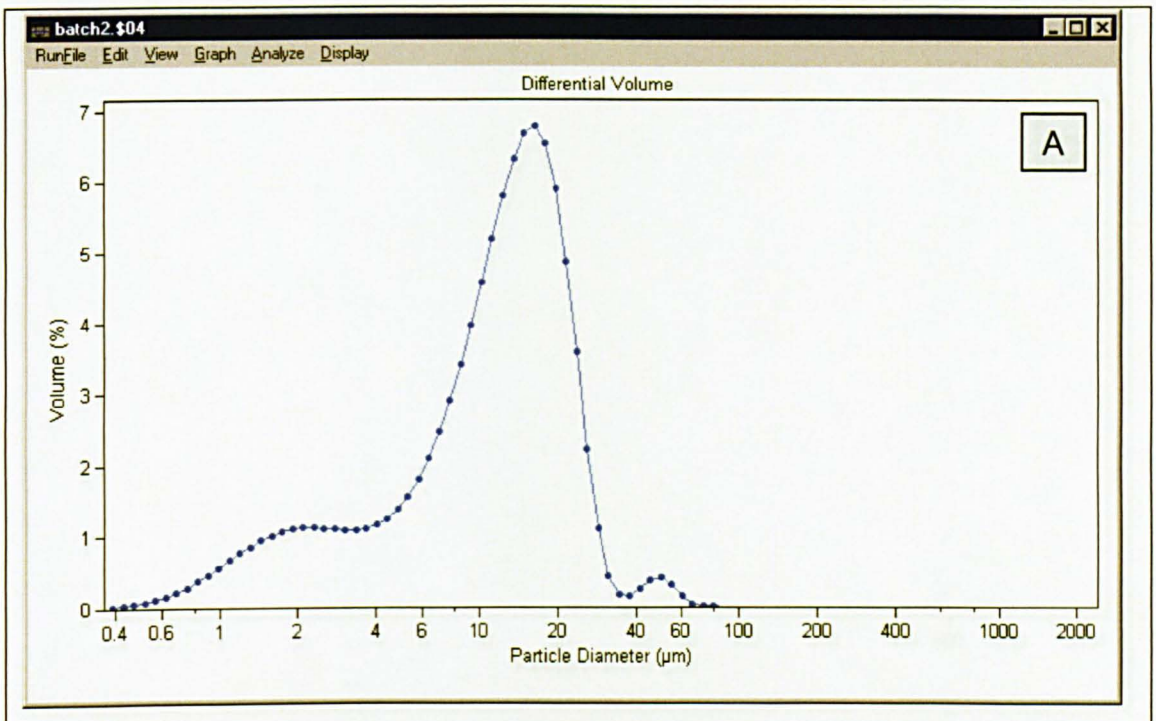
Both the milling method and the emulsion method were tried as a means of producing microparticles. Milling failed to produce a substantial proportion of particles smaller than 50 μm, with >90% of PLGA ending up in larger fractions. The emulsion method resulted in smaller particles and therefore adopted as the method of choice.

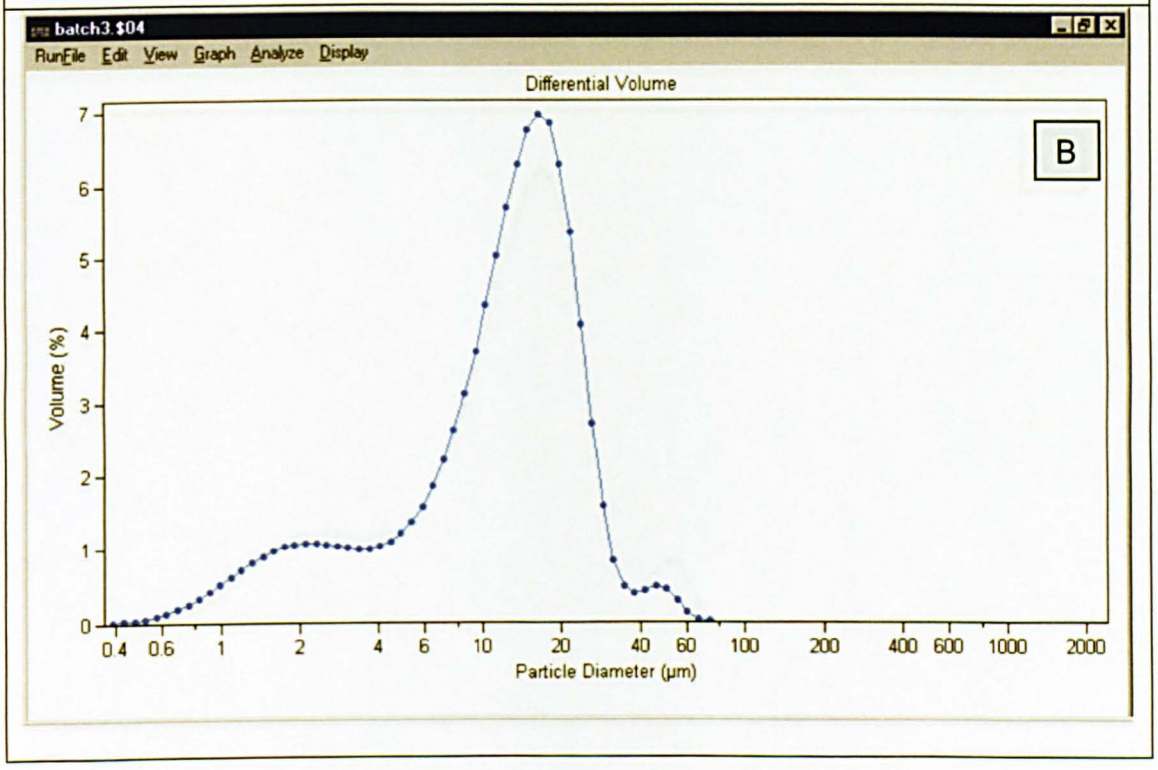
Table 10.3.1.1 lists the mean, median, and range of particle sizes obtained in different batches as assessed by the laser diffraction method, with Fig 10.3.1.1. showing the particle size distribution graphically and Fig 10.3.1.2 showing SEM images of the microparticles. In these batches, the mean particle size obtained was 12.31 μm, median 11.70 μm and mode 15.37 μm, with corresponding standard deviations being 1.13, 1.06, and 1.58, respectively, and the coefficients of variation 9%, 9% and 10%, respectively.

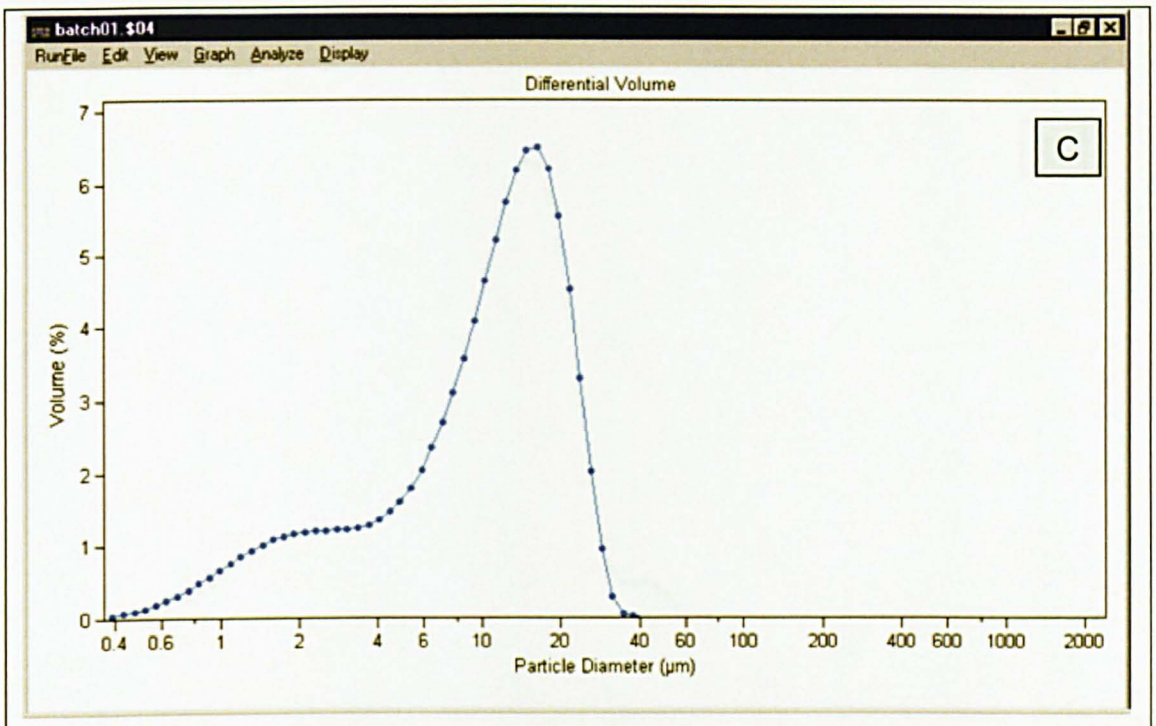
Batch	Mean μm	Median μm	Mode μm	Minimum μm	Maximum μm
A	12.79	12.24	16.40	0.38	76.43
B	13.59	13.03	16.40	0.38	69.62
C	11.58	11.38	16.40	0.38	36.24
D	13.46	12.25	16.40	0.45	69.62
E	12.29	11.50	14.94	0.38	69.62
F	11.15	10.23	12.40	0.50	43.66
G	13.08	12.74	16.40	0.38	69.62
H	10.51	10.24	13.61	0.38	33.00
Average	12.31	11.70	15.37	0.40	58.48

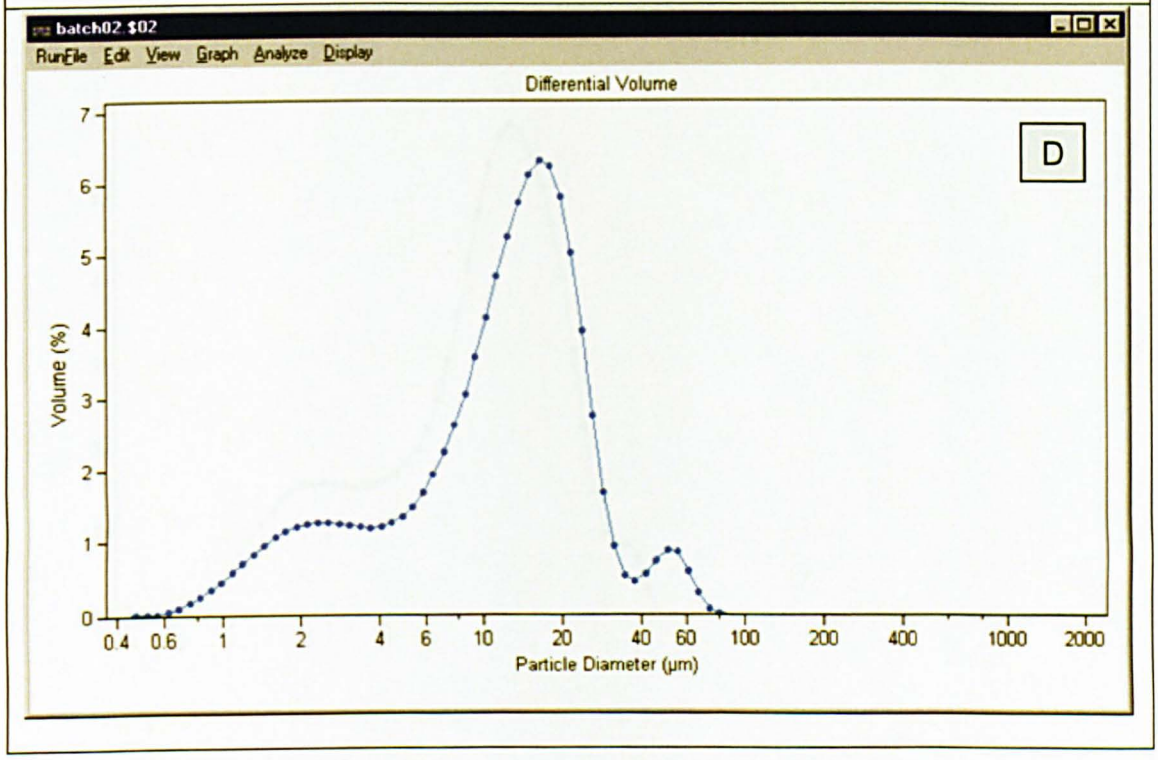
Table 10.3.1.1: Sizes of microparticles obtained in different batches.

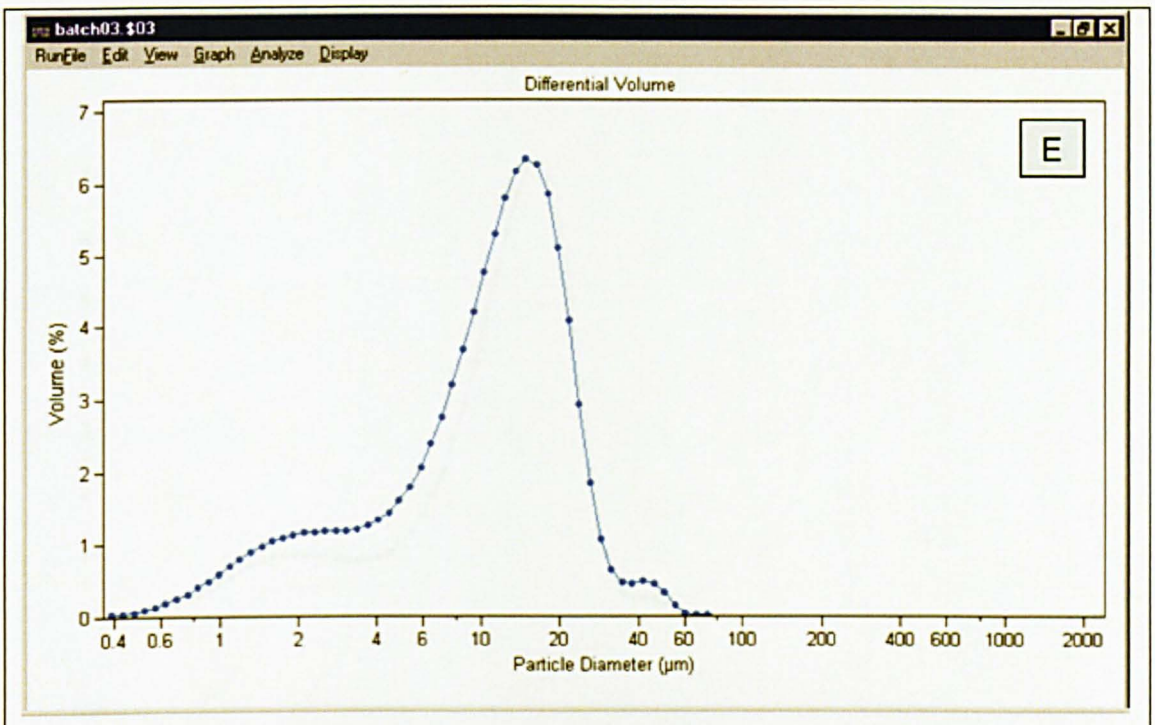
Fig 10.3.1.1 (following pages): Distribution of PLGA particle size obtained by different batches, showing particle size (μm) on horizontal axis and the volume (%) of particles that are of that size on the vertical axis

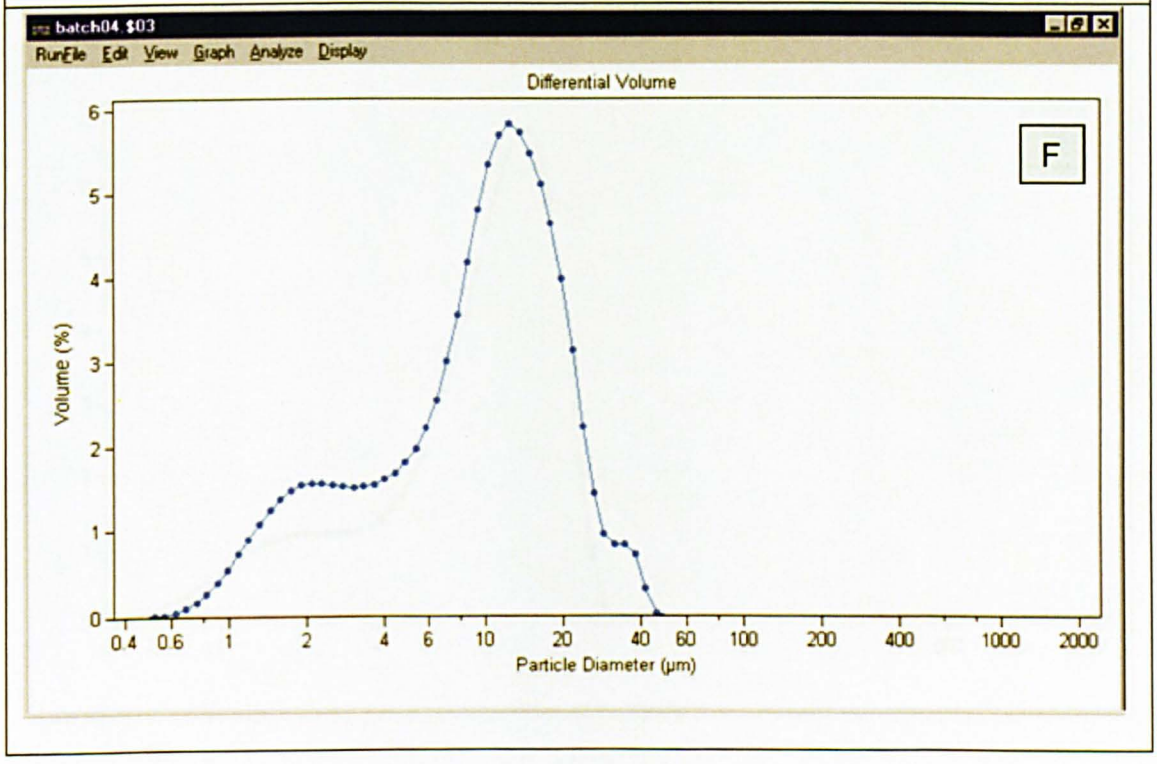

batch2.$04
RunFile Edit View Graph Analyze Display
Differential Volume
A
Volume (%)
0 1 2 3 4 5 6 7
0.4 0.6 1 2 4 6 10 20 40 60 100 200 400 1000 2000
Particle Diameter (µm)

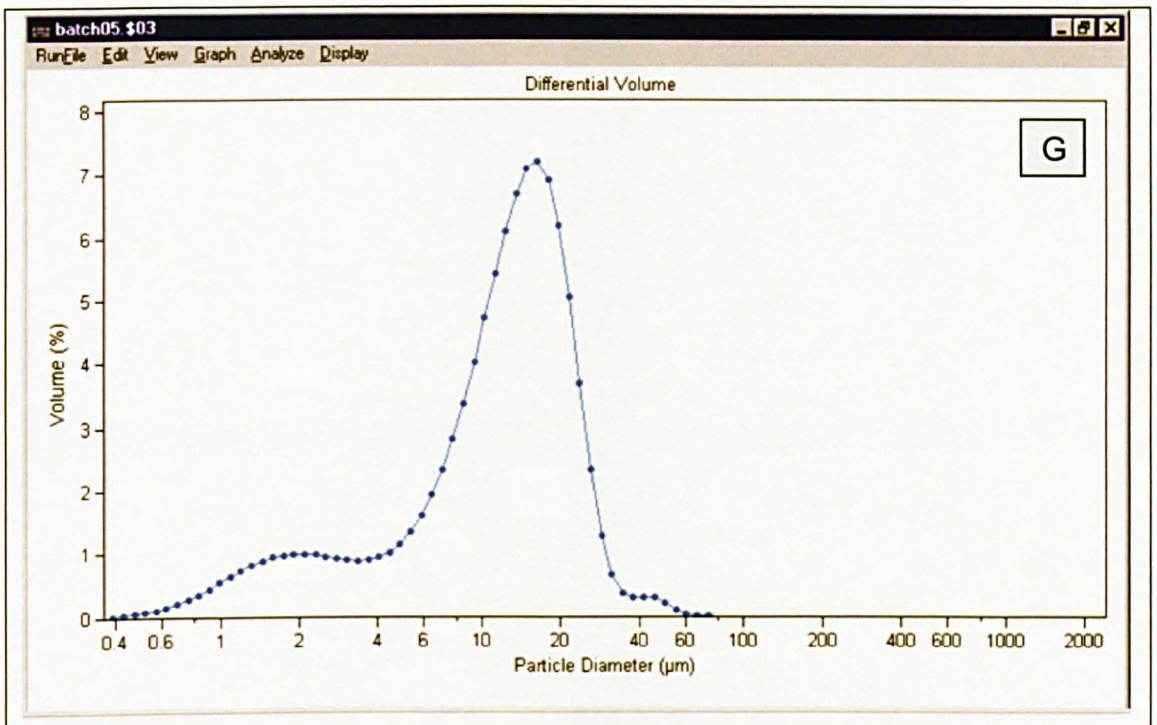

batch3.$04
RunFile Edit View Graph Analyze Display
Differential Volume
B
Volume (%)
0 1 2 3 4 5 6 7
0.4 0.6 1 2 4 6 10 20 40 60 100 200 400 600 1000 2000
Particle Diameter (µm)

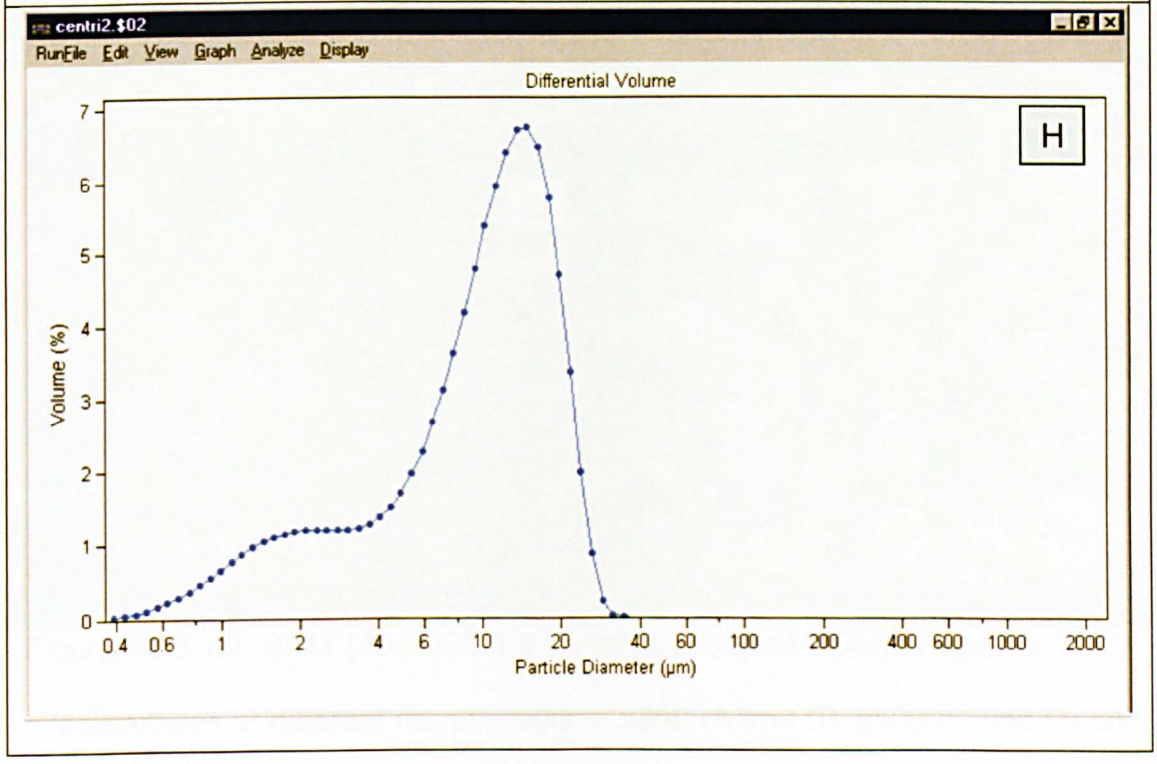

batch01.$04
RunFile Edit View Graph Analyze Display
Differential Volume
C
Volume (%)
0 1 2 3 4 5 6 7
0.4 0.6 1 2 4 6 10 20 40 60 100 200 400 600 1000 2000
Particle Diameter (µm)


batch02.$02
RunFile Edit View Graph Analyze Display
Differential Volume
D
Volume (%)
0 1 2 3 4 5 6 7
0.4 0.6 1 2 4 6 10 20 40 60 100 200 400 1000 2000
Particle Diameter (µm)


batch03.$03
RunFile Edit View Graph Analyze Display
Differential Volume
E
Volume (%)
0 1 2 3 4 5 6 7
0.4 0.6 1 2 4 6 10 20 40 60 100 200 400 600 1000 2000
Particle Diameter (µm)


batch04.$03
RunFile Edit View Graph Analyze Display
Differential Volume
F
Volume (%)
0 1 2 3 4 5 6
0.4 0.6 1 2 4 6 10 20 40 60 100 200 400 1000 2000
Particle Diameter (µm)


batch05.$03
RunFile Edit View Graph Analyze Display
Differential Volume
G
Volume (%)
0 1 2 3 4 5 6 7 8
0.4 0.6 1 2 4 6 10 20 40 60 100 200 400 600 1000 2000
Particle Diameter (µm)


centri2.$02
RunFile Edit View Graph Analyze Display
Differential Volume
H
Volume (%)
0 1 2 3 4 5 6 7
0.4 0.6 1 2 4 6 10 20 40 60 100 200 400 600 1000 2000
Particle Diameter (µm)

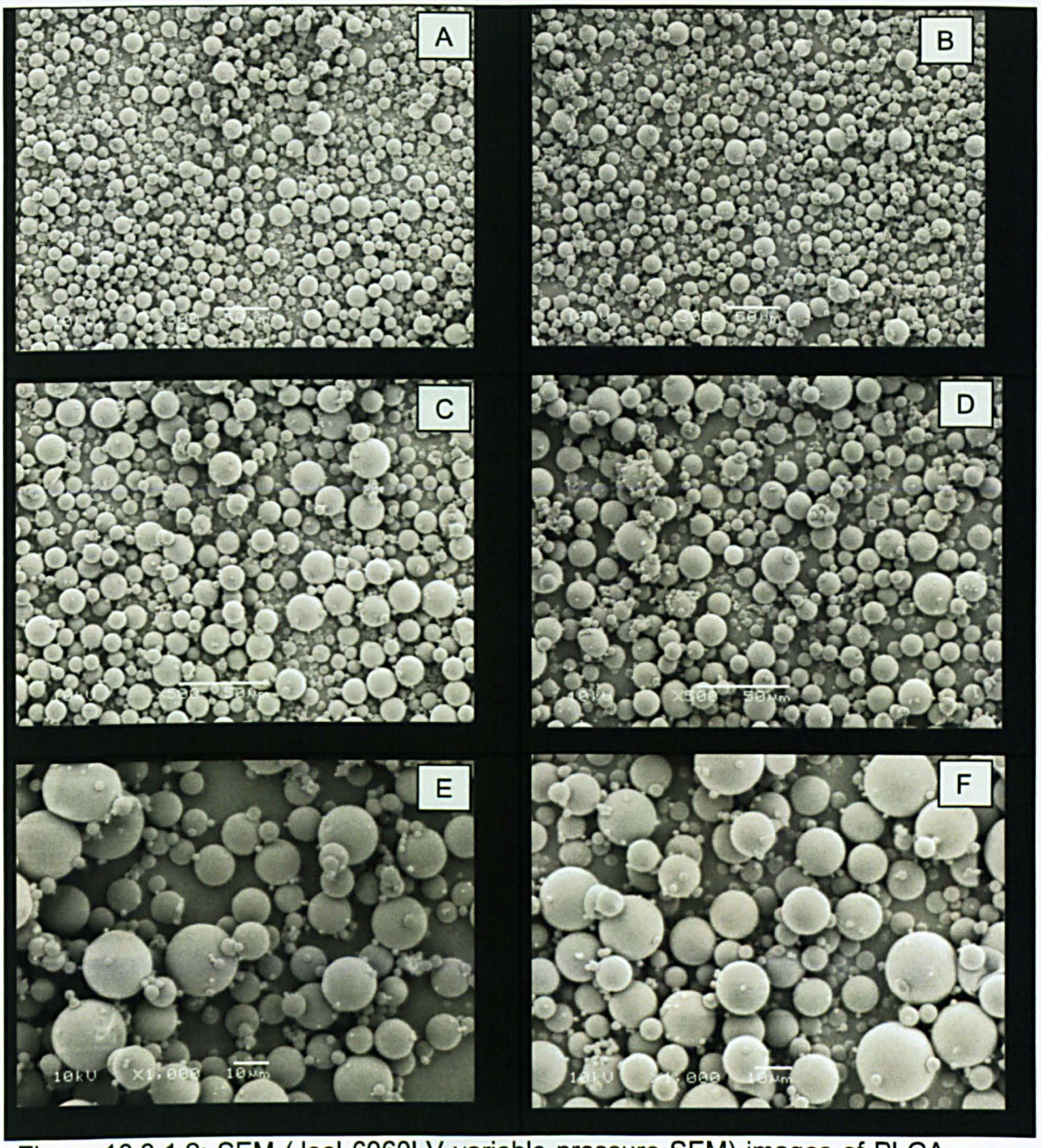


Figure 10.3.1.2: SEM (Jeol 6060LV variable pressure SEM) images of PLGA microparticles at different magnifications: x300 (A and B), x500 (C and D) and x1000 (E and F)

10.3.2 Antibiotic pellets

The pellets were prepared as detailed in the main methods section, using a mould that was specifically designed and manufactured for this purpose by the Medical Physics Department at The University of Nottingham. The process is summarised in Fig 10.3.2.1, with photographs of pellets shown in Fig 10.3.2.2. The pellets measured 3 mm in the long axis and 2.5 mm in diameter, with a central 1 mm diameter hole. Using an inactive pellet prepared with high viscosity CMC as an example, the calculated mean density was 0.829 g/cm^3, with 33.66% porosity.

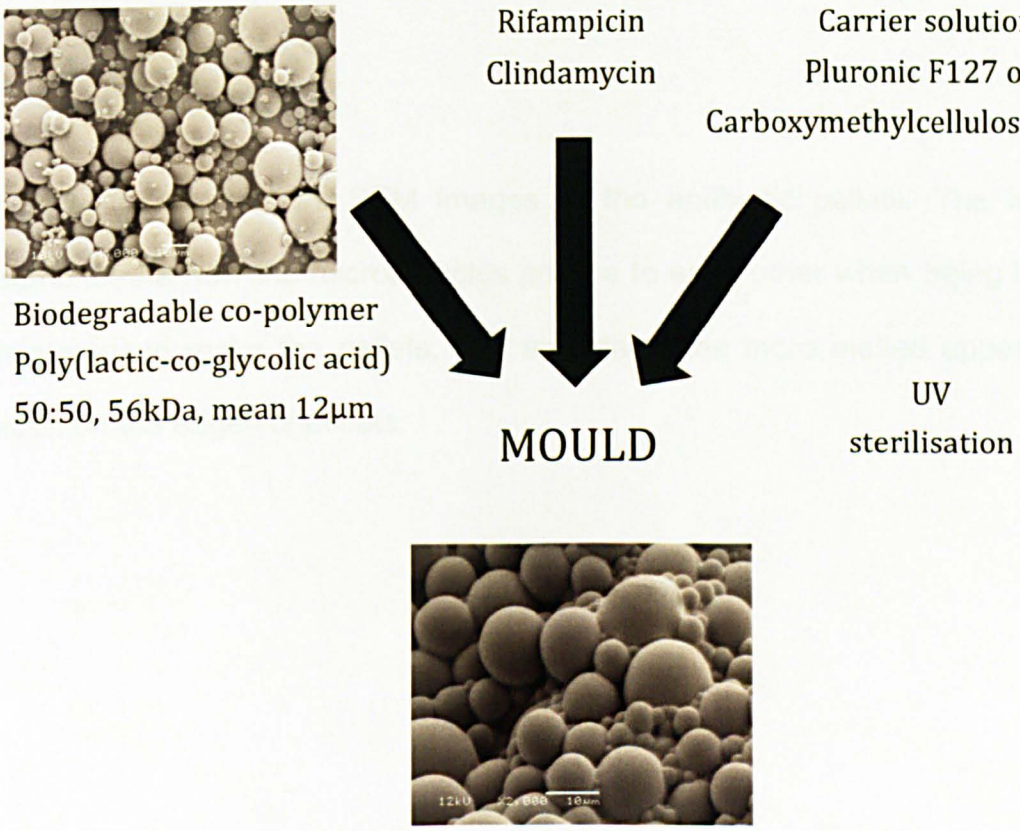


Fig 10.3.2.1: Diagrammatic summary of antibiotic pellet production, showing an SEM image of PLGA microparticles prior to sintering (top) and the pellet after sintering (bottom)

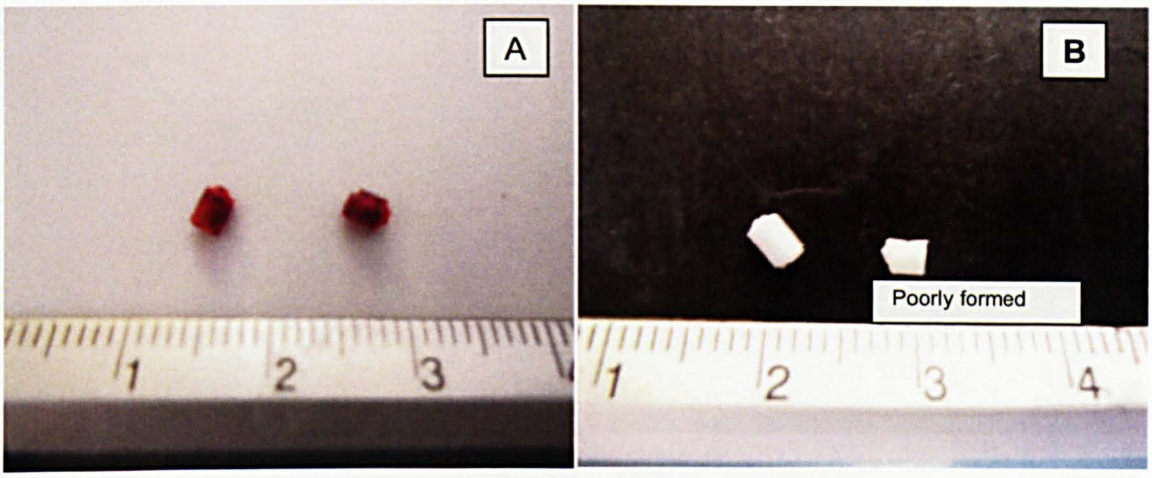


Figure 10.3.2.2: Photographs of the antibiotic pellets. Image A shows pellets containing antibiotics, coloured red by rifampicin. Image B shows pellets without antibiotics; one is well formed, but the other one has not been formed properly and had to be discarded.

Figure 10.3.2.3 shows SEM images of the antibiotic pellets. The images demonstrate how the microparticles adhere to each other when being heated (sintered) to make the pellets, and also show the more melted appearance seen on the edges of pellets.

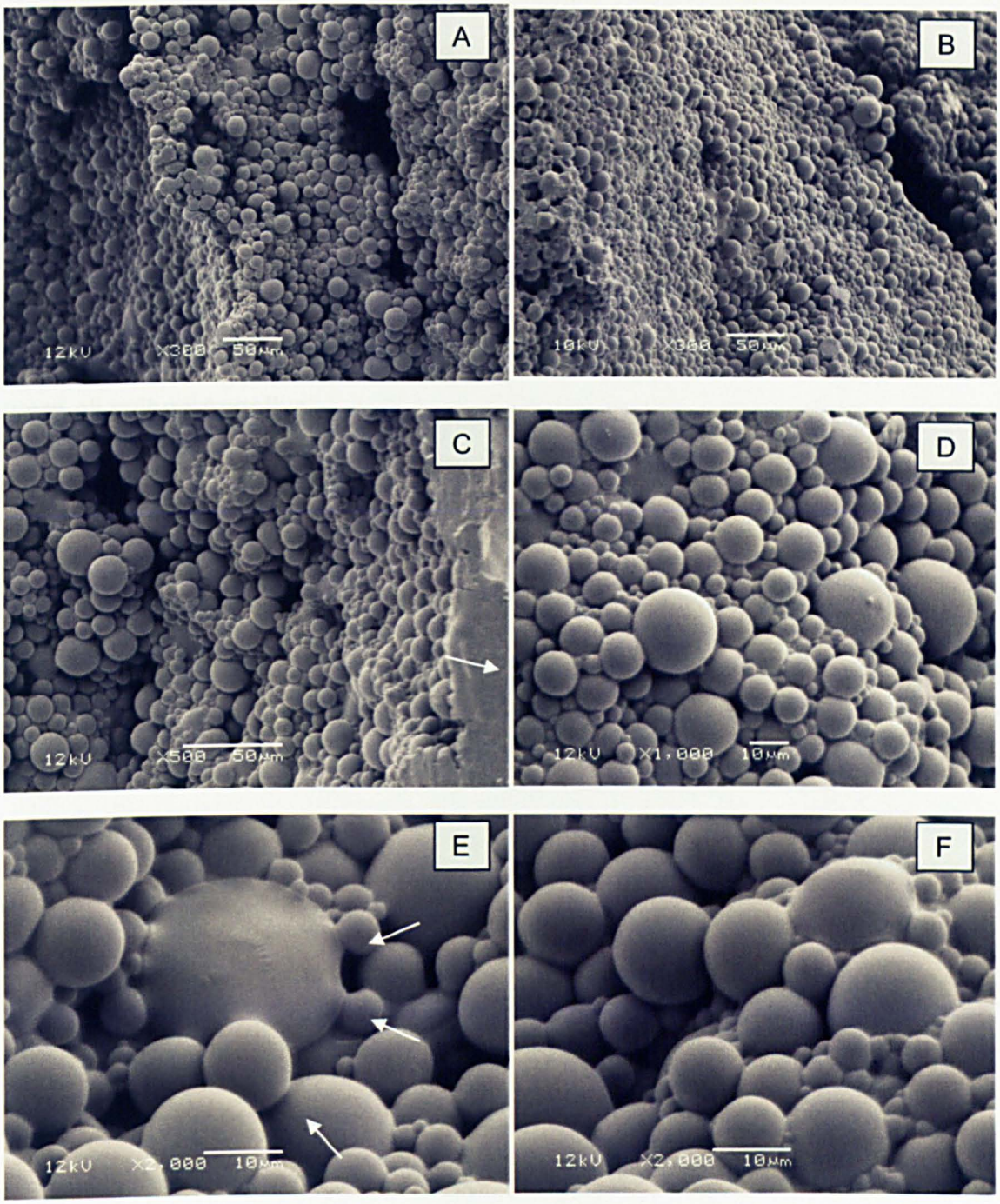


Figure 10.3.2.3: SEM (Jeol 6060LV variable pressure SEM) images of antibiotic pellets at different magnifications: x300 (A and B), x500 (C), x1000 (D) and x2000 (E and F). Arrow in C illustrates the melted edge of the pellet where microparticles had lost shape and formed an amorphous mass. Arrows in E illustrate individual microparticles adhering to each other.

10.3.3 Sterilisation

Sterilisation for the purposes of this study was achieved by exposure of the pellet components to UV light for 15 minutes prior to mixing, and again for 15 minutes once the pellet has been sintered and formed. Pellets (without antibiotics) prepared in this manner were placed into TSB for 5 days, and the resulting TSB plated onto Sheep Blood Agar. The samples tested in triplicate were all culture negative.

10.4 DISCUSSION

10.4.1 Surgical considerations

The development of the antibiotic pellet needs to incorporate several important surgical characteristics. The middle ear is only in the region of 2 mL in volume, and therefore the device would need to be small, and capable of being passed through a standard myringotomy incision used to place VTs. As the device would be placed onto the promontory of the middle ear at time of myringotomy and VT insertion, it was felt that it should be no larger than the shaft of a standard VT. Therefore the device dimensions chosen were an outer diameter of 2.5 mm and a length of 3 mm.

Due to concerns about possible Eustachian tube obstruction, it was decided that the centre of the device should be perforated with a 1 mm central void, to allow air through the device should it move onto the Eustachian tube orifice. This gave the final device the appearance of a hollow tube. In reality, Eustachian tube obstruction would be unlikely for several reasons. The antibiotic device would be too heavy to be moved by ciliary action from the position of placement on the promontory. Clinical experience would suggest that it would be unlikely to move, because any prostheses placed in the middle ear, and the occasional VT that moves from the ear drum to fall into the middle ear, do not "float" around despite little or no fixation. Further, the pellet itself is porous, whilst the presence of a functional VT itself allows aeration of the middle ear, thus rendering Eustachian tube function actually unnecessary.

An alternative to a pellet sitting in the middle ear would be to have the pellet attached to the medial aspect of the ventilation tube that is within the middle ear. That would eliminate any problems associated with pellet movement in the middle ear. However, the tube sits in the ear drum, but the biofilms are likely to be more numerous on the promontory on the medial wall of the middle ear, rather than at the lateral wall of the middle ear where the ear drum is. Therefore, a strategy that attaches the antibiotic to the ventilation tube would rely much more on adequate transport of the antibiotic throughout the middle ear.

Another alternative would be to use an injectable formulation (Lane, Okumu and Balausubramanian 2008) that is liquid at room temperature but solidifies at body temperature after injection. This would remove the need to make an incision in the ear drum if the injectable formulation can be administered with a needle. The downside of this would be the difficulty in controlling exactly where the formulation ends up, with a tendency of the paste to run into dependent areas before solidifying. Also the ear drum incision itself is a small procedure, and an injection is only marginally safer, with both still needing a general anaesthetic. However, the disadvantages of a VT being in place (for example water avoidance) would be avoided.

Movement of antibiotic throughout the effusion and mucin is important if the antibiotic pellet strategy is to work. The antibiotic will sit in one part of the middle ear, but biofilms may be sited away from where the pellet is. We have

found biofilms in the effusion in OME as described Chapter 7, but this does not tell us which part of the middle ear the biofilms reside in, because the effusion is aspirated from the middle ear as a whole. The studies on middle ear mucosal biopsies that found biofilms on the mucosa (Hall-Stoodley, et al. 2006) sampled the promontory / Eustachian tube area, which is on the medial wall of the middle ear (see Fig 2.1.2). However, the biofilms could reside anywhere in the middle ear cavity, or within the mastoid air cells (a connecting network of air cells within the temporal bone, extending from the middle ear and going posteriorly). Certainly, an effusion is often encountered in the mastoid air cells if one operates on them, but a child having mastoid surgery would not be having this purely for OME and will have additional pathology so that such circumstances cannot be said to be representative of OME. It is not known where the biofilms do or do not reside: biopsy studies are limited to the promontory and the medial wall of the middle ear, because this is the only area that can be accessed during ventilation tube insertion; accessing other areas for biopsy would involve much greater surgery than a simple incision of the ear drum for tube insertion, and would therefore be unethical.

Does it matter where in the middle ear the biofilm is? If the antibiotic can travel through the effusion or the mucus layer from the pellet into the rest of the middle ear, and reach high enough antibiotic levels at sites distant from the pellet, then the pellet strategy could be successful irrespective of where the biofilm is. The pellet is likely to sit near the Eustachian tube orifice, in the lower part of the tympanic cavity and adjacent to the anterior part of the promontory; the pellet will sit there partly due to gravity and partly because

this is the area surgically accessible at the time of ventilation tube insertion. Any bacteria reaching the middle ear to cause glue ear come up the Eustachian tube; therefore it is reasonable to think that the area where the pellet will sit is the area first exposed to bacteria, although one cannot assume that the bacteria will stop there and not travel further into the mastoid air cells. This is also the area with the greatest number of goblet cells (Bak-Pedersen and Tos 1976) (Tos and Bak-Pedersen 1976) that produce the effusion.

If the biofilms are sited away from the promontory, and the antibiotic cannot travel through the mucin then the pellet strategy would not be successful, unless modifications are incorporated to increase diffusion of drug through the mucus (Cu and Saltzman 2009). Section 10.4.6 has addressed the issue of antibiotic diffusion, including a research strategy as to how this may be investigated. However, the observation that levels of at least some antibiotics in the middle ear effusion approach those seen in plasma suggests adequate diffusion of antibiotics into and through the middle ear effusion is possible (Sundberg, et al. 1979) (Thorn 1987). For example, cefaclor (Ernstson, et al. 1985) and erythromycin (Sundberg, Eden and Ernstson 1982) reach levels above MIC of common pathogens, and clarithromycin levels in the effusion are actually higher than those in plasma (Sundberg and Cederberg 1994).

Cilia in the middle ear beat towards the Eustachian tube, with the physiological intention of clearing mucus down the Eustachian tube (Kubba, Pearson and Birchall 2000). Although the cilia would not be strong enough to move the pellet itself, they may propel any antibiotic in the effusion / mucus

towards the Eustachian tube thus interfering with the uniform distribution of antibiotic. However, unlike in health, in OME the effusion is very viscous to the extent that the cilia are unable to move the effusion (Smirnova, et al. 2002) (Kubba, Pearson and Birchall 2000). In relation to the antibiotic pellet, the ciliary action is thus likely to be unimportant in the presence of a thick effusion, but it may be an important factor where there is no effusion either because it has all been aspirated when the ventilation tube was inserted or because normal health of the middle ear is restored.

10.4.2 Production of microparticles

There are four main ways of producing microparticles (Li and Jasti 2006) (Svenson 2004):

- Milling
- Emulsion solvent evaporation method, using oil-in-water, water-in-oil, or double emulsion water-in-oil-in-water
- Phase separation process, where particle formation is triggered by the addition of an anti-solvent to a solution
- Spray drying, where a solution is passed through a nozzle to break up into droplets, followed by drying

We had chosen to focus on small microparticles (smaller than 50 μm), on the basis that a device composed of small particles would have a greater number of channels and pores and thus theoretically better modified release properties (the illustration in Fig 10.4.2.1 is diagrammatic only, as a variety of different ways of sphere packing exist (Sowa, Koch and Fischer 2003));

conversely, in a device composed of larger particles the number of gaps / channels would be less (although each gap is larger) with a greater tendency to release a large amount of antibiotic all at once in a less controlled fashion. The size of the particles chosen was also partly guided by other work in our departments. Concurrent projects are investigating a PLGA matrix / hydrogel modified release device using particles between 100 and 200 μm, and this releases antibiotics for up to 3 weeks; however, the size of the device being investigated is much larger than the proposed middle ear pellet, and using particles of 100 to 200 μm for the smaller OME pellet was thought to be unlikely to achieve a sufficiently high antibiotic release level for the required duration.

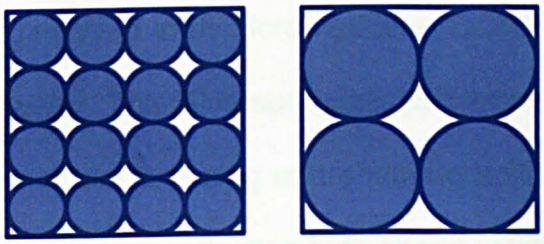


Fig 10.4.2.1: A device composed of many smaller particles (A) has a greater number of pores than a same size device composed of larger particles (B).

Although the milling method was initially investigated to produce microparticles, this proved unsatisfactory as most of the PLGA ended up in particles of larger sizes. The advantage of the milling method would have

been avoidance of solvents. The emulsion methods use solvents, and therefore when planning any future clinical trials one would have to ensure that no trace of potentially toxic solvents remained. Similar considerations apply to the phase separation process, and also to spray-drying because the PLGA would still have to be emulsified prior to spray-drying (Gavini, et al. 2005). A future commercial venture may involve investigation of the milling method again, because large industrial scale milling at low temperature may be able to produce small microparticles by milling for longer times (using low temperature to prevent over-heating of the temperature-sensitive polymer). For the purposes of this study the milling method did not achieve the desired particle size, and therefore the emulsion method was adopted.

An emulsion is a mixture of two immiscible liquids, in this case PLGA dissolved in dichloromethane (DCM) and polyvinyl alcohol (PVA) dissolved in water. The Silverson mixer (Silverson 2010) used to make the emulsion has a multistage mixing and shearing action (Fig 10.4.2.2): the liquid is drawn into the work-head using high-speed rotating blades, and the centrifugal force then drives the particles to the periphery of the work-head where they are subjected to a milling action between the rotating blades and the perimeter of the work-head. The material is then forced out of the work-head through the surrounding perforations at high speed under intense hydraulic shear forces. As the mix is expelled from the head to the periphery of the main volume of the emulsion, fresh material is drawn into the work-head, creating a continuous mixing cycle.

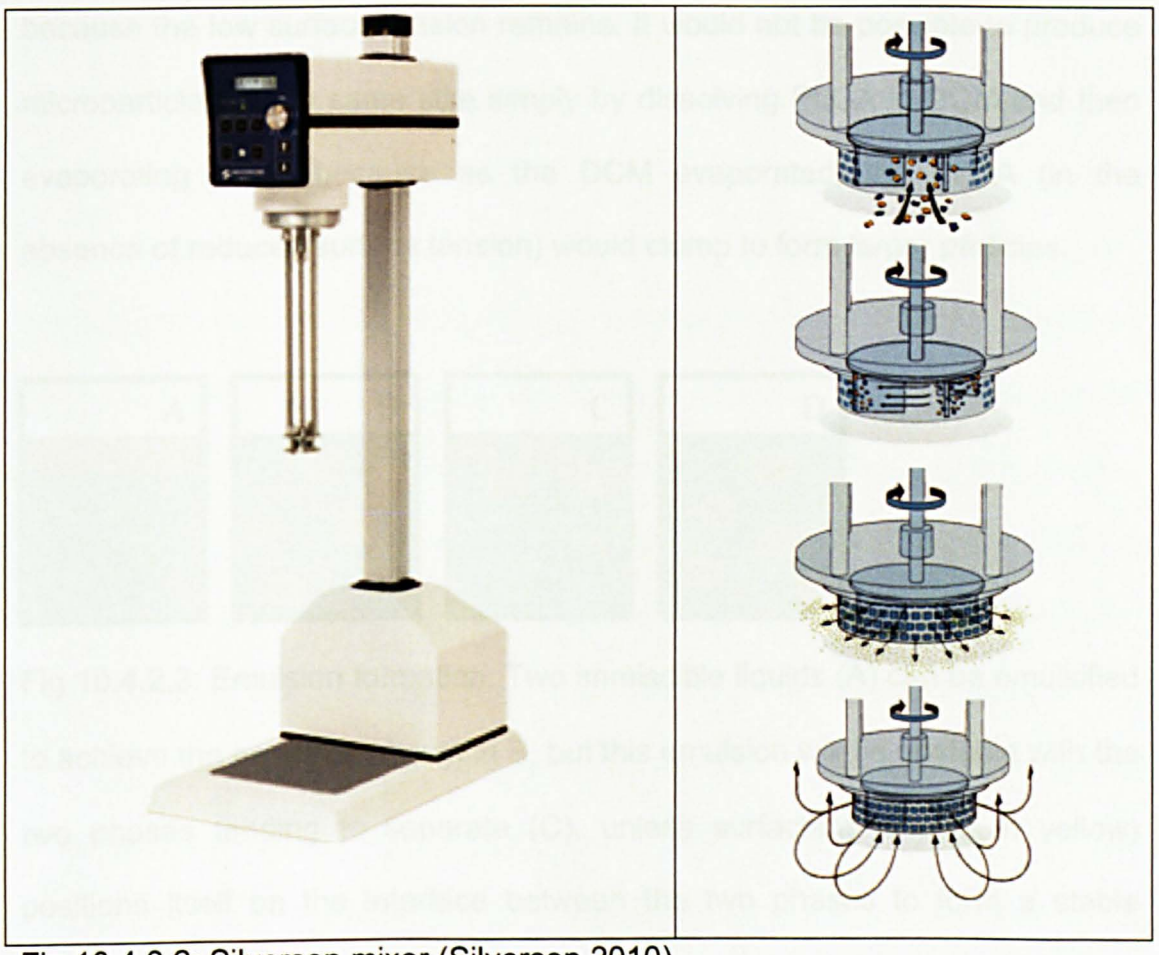


Fig 10.4.2.2: Silverson mixer (Silverson 2010).

The Silverson mixer produces the emulsion, with individual small PLGA microparticles in DCM (oil phase) dispersed in the aqueous PVA. The large volume of PVA relative to the DCM / PLGA solution means that PLGA particles end up being widely dispersed, whilst the emulsifying properties of PVA mean that the PLGA particles have reduced surface tension and remain dispersed in a stable emulsion without clumping, and the oil phase of the emulsion does not separate from the aqueous phase (different emulsion stages are shown in Fig 10.4.2.3). The emulsion is then stirred overnight so that the DCM evaporates, and the PLGA microparticles remain widely spaced in PVA solution. Once the PVA is removed and the particles filtered and

washed with water, the microparticles do not clump into larger particles because the low surface tension remains. It would not be possible to produce microparticles of the same size simply by dissolving PLGA in DCM and then evaporating DCM, because as the DCM evaporated, the PLGA (in the absence of reduced surface tension) would clump to form larger particles.

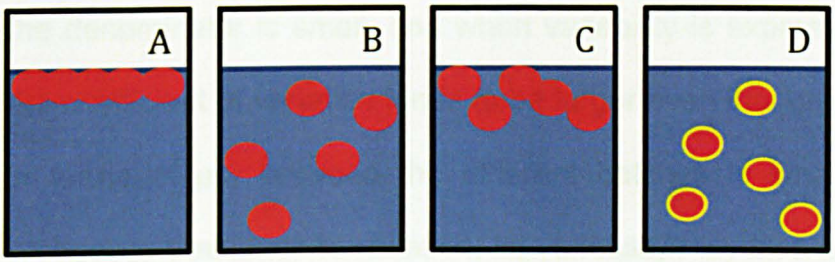


Fig 10.4.2.3: Emulsion formation. Two immiscible liquids (A) can be emulsified to achieve the emulsion shown in B, but this emulsion will be unstable with the two phases tending to separate (C), unless surfactant (shown in yellow) positions itself on the interface between the two phases to form a stable emulsion (D).

Obtaining the desired particle size during manufacture is dependent on a number of parameters that can be varied during manufacture (Herrmann and Bodmeier 1998) (Tsukada, et al. 2009), including ratio of polymer to oil phase and aqueous phase, emulsification method, stirring rate and time, and temperature. In these experiments, existing particle preparation protocols were adapted, and the size of the particles obtained checked to ensure reproducibility. The mean particle size calculated over several batches was 12.31 µm, the median being 11.70 and mode 15.37 µm. A number of smaller particles was also obtained, as was a small number of larger particles.

The coefficient of variation, a measure of the variability and reproducibility calculated by dividing the standard deviation with the mean, in this study was 9 – 10%. Although this variability between different batches may instinctively sound high, one needs to consider that this statistical measure is dependent on the magnitude of the actual measurement in question (McLaughlin, Aitchison and Macfarlane 1998). If one is measuring small microparticles then the denominator is small, and when variability is expressed as a percentage the coefficient of variation tends to be larger even though the actual difference in terms of μm between the different batches is small. It is not just the production that leads to variation, as variability may also arise during sampling of the microparticles for size analysis (Saleem, Donovan and Smyth 2007), and during size analysis itself. Generally, coefficients of variation in the region of 10% are considered fair, 20% is poor and 5% is good (Barringer 1998), but allowing for the small actual particle size (and thus small variations in size) in this study the variability seen was deemed satisfactory for our purposes. However, future work for clinical application should ideally aim to reduce any batch to batch variation.

10.4.3 Sizing of microparticles

A number of different methods of particle sizing are available (Saleem, Donovan and Smyth 2007), but the traditional techniques of sieving or sedimentation have been largely replaced by modern methods taking advantage of new electronic and computer technology. The two most commonly used methods of particle sizing are light diffraction analysis and the

electrical sensing zone method (Beckman-Coulter 2010) (Xu and Di Guida 2003).

The diffraction method commonly uses a laser, and determination of particle size relies on the interaction of particles with the laser beam. If a particle is struck by light of a wavelength much larger than the particle itself then scattering of light occurs at very small angles, but if the particle is larger compared to the wavelength of the light then the scattering angle is greater. The intensity of light reaching the detectors positioned around the sample at various scattering angles is thus related to the particle size, so that size and the distribution of different sizes can be calculated.

In the electrical sensing zone (ESZ) method, also known as the Coulter method, the particles are suspended in an electrolyte solution and the liquid passes through a small orifice across which electrical conductivity is measured. When individual non-conducting particles pass through the orifice the electrical conductivity is momentarily interrupted, and the degree of interruption relates to particle size, with the number of separate interruptions counted to give the size distribution.

In addition to laser diffraction and ESZ a variety of other methods are also used (Saleem, Donovan and Smyth 2007). Photo analysis and optical counting involves taking a photomicrograph (scanning or transmission electron microscopy) of the sample to determine particle size; however, this measures only a small number of particles in each image, although automated

photography and counting software are available for large volume work. Other methods (Saleem, Donovan and Smyth 2007) include analytical ultracentrifugation that allows real time monitoring of the sample with an optical detection system such as ultraviolet light, small angle X ray scattering, and the suspension separation technique of field flow fractionation where the flowing suspension is influenced by a perpendicular field, for example electrical, magnetic, thermal or gravitational.

The great advantage of light diffraction and ESZ is their simplicity and relative automation, with reduced reliance on human expertise and therefore potentially fewer errors at least in inexperienced hands. Both methods are commonly used to size particles (Beckman-Coulter 2010) (Xu and Di Guida 2003), but have different properties. Laser diffraction assumes that particles are spherical; the particle is seen as a two-dimensional object and the size is reported as a function of the cross-sectional area; in the case of spheres the cross-sectional area is the same irrespective of orientation of the sphere. However, in the case of rod- or plate-shaped objects, the cross-sectional area will depend on the orientation of the object as it is hit by the light, so laser diffraction will produce generally smaller mean values with a large size distribution than the ESZ method which is not affected by the orientation of the particles. However, if a sample is porous, then the ESZ method may undersize particles because the pores are filled by the conducting medium and therefore not detected as a change in conductance (Michoell, et al. 1994). Understanding that the two methods behave differently allows one to choose

the appropriate method, and aids in particle characterisation (Beckman-Coulter 2010).

The laser diffraction method was used in the work presented here, as the microparticles were spherical. If the ESZ method had been used, the sizing would be likely to be very similar as the two methods give comparable results in the case of spherical particles (Beckman-Coulter 2010).

10.4.4 Production of pellets

The pellets were produced in a specially designed PTFE and stainless steel mould. The mould consisted of two parts, a base PTFE plate containing upstanding stainless still pins (to create the central gap in the pellet), and a top PTFE plate with a series of holes, each 2.5 mm diameter and 3 mm deep. Whilst adequate for this study, a number of problems were encountered with this method. The process of pellet manufacture was laborious, as each pellet was made individually by hand, with potential variability. The small quantities of PLGA, antibiotic and carrier involved, even when making numerous pellets at once, meant that weighing them accurately was difficult, with potential for errors. The mixture was mixed by hand, the paste was placed into the mould using a spatula, compaction of the paste into wells was done manually, and the pellets were taken out of the mould by hand; all these steps were open to day-to-day variation and operator differences. A proportion of pellets were noted to be incorrectly formed due to poor compacting of mix into the wells (see Fig 10.3.2.2B for example), and had to be discarded. Operator variability

was also noted, and a learning curve existed before good quality pellets could be reliably produced.

Although the manufacture of pellets in this manner was suitable for testing a variety of different pellets to determine which may be the best for further investigation, any future clinical use would require improvement. The fact that pellets of only one type would be made in large quantities would make it easier, and a single person could be designated for the manufacture to eliminate operator bias, or the process automated for example by automatic mixing or application of identical compacting pressure to identical mix weight each time. The mould could also be improved. For example, instead of using a pin on a base plate and a hollow top plate, the mix could be compacted into a plastic tube with a central wire (to give the central gap in the pellet), and after sintering the tube cut into desired lengths prior to peeling the tube off the pellet. If the product approaches a commercial stage, it is likely that a company would be required to develop an automated manufacturing process that would ensure the consistent production of a large number of identical pellets, with quality controls in place to check the process. The effect that storage has on the antibiotic pellets would also have to be investigated, as this study used pellets prepared no more than a week in advance but clinical use (and convenience) is likely to require storage for a much longer time period.

10.4.5 Choice of modified release device

The concept of a modified release antibiotic formulation for eradication of glue ear biofilms represents a novel idea that may improve OME treatment and

outcome. Therefore, whether a drug release device would work, and what it should consist of was not known. The experiments described in the subsequent chapters thus represent an exploration of a new idea, with the aim of guiding future experiments that could refine the product. However, as the idea is a new one, a single modified-release method had to be chosen for use in the exploratory experiments. The guiding principle was to focus on a product that would be safe and easily adapted, and ideally made from components already in clinical use so that any laboratory benefits could be translated into patient use as speedily as possible.

PLGA

PLGA is one of the commonest studied components of biodegradable systems (Bossy, et al. 2008), with a long history of safe clinical use in sutures, implants, prosthetic devices, and depot drug delivery systems. PLGA is generally considered to be very safe: studies examining toxicity by implanting PLGA *in vivo* show only a very localised granulomatous response with minimal granulation tissue and fibrosis (Anderson and Kim 1984). PLGA is approved by the US Food and Drug Administration, and this strong safety record makes it an ideal starting point for developing new treatments for OME. The different types of PLGA have different ratios of lactic to glycolic acid, and this affects the rate of degradation (Bossy, et al. 2008); generally speaking a higher lactic acid content and higher molecular weight lead to slower degradation, but PLGA 50:50 has the fastest degradation time (about two months) and this was chosen for these experiments as a longer drug release was not required.

In addition to PLGA, poloxamer or CMC were used as a gel that carried the antibiotics. Gels or solutions alone would not be suitable as they would dissipate down the Eustachian tube, and a three-dimensional structure that would physically sit in the middle ear was therefore required. On the other hand, a PLGA matrix alone would also not be suitable in the absence of a modified-release gel filling the pores (Anderson and Kim 1984).

Monolithic drug delivery systems

A variety of different drug delivery systems are available, as discussed in the introductory chapter. The system chosen, antibiotics mixed with gel within a PLGA scaffold, is an example of a monolithic system where the drug is homogeneously distributed throughout the system. Monolithic systems have several advantages (Anderson and Kim 1984):

- Drug delivery rate can be controlled simply by changing the drug loading
- Lifetime of the device is easy to control
- Physical dimensions can easily be altered
- A wide variety of agents can be delivered
- They are easy to manufacture
- There is no risk of drug being released all at once (dumped) unlike the possibility of system rupture in reservoir systems

It is for these reasons that PLGA-based monolithic formulations were chosen for the preliminary studies of OME biofilm eradication. In the context of OME biofilms, being able to build a pellet of the correct size for the middle ear is

crucial. Similarly, the pellet will sit in a potentially infected environment, so once antibiotics are released the polymer degradation should ideally be complete soon afterwards; again, PLGA allows this to be tailored. PLGA monolithic devices also deliver a wide variety of agents, important when considering several antibiotics, and the ability to increase antibiotic release simply by increasing loading is very attractive when a variety of different formulations releasing high antibiotic levels are desired.

PLGA monolithic devices undergo bulk, rather than surface degradation: they do not gradually reduce in size by releasing PLGA from the surface, but instead the device is penetrated by water at the start, with degradation due to hydrolysis occurring both on the surface and in the core of the matrix. Therefore, in addition to erosion, drug release is also controlled by drug diffusion, and because of this the drug release can be unpredictable and is not easily modelled (Rosoff 1989). One of the major disadvantages of PLGA monolithic devices is the difficulty in achieving zero-order drug release, where the release of drug is independent of the amount of drug remaining in the device, i.e. the same dose is released every day. Because diffusion affects drug release, the amount of drug remaining in the system affects drug release, so that typically much drug is released early, and less drug is released in the later stages (Anderson and Kim 1984).

How does this release pattern fit with the proposed biofilm eradication strategy? In the previous chapter that investigated biofilm treatment with antibiotics, eradication was possible using a high antibiotic level over several

weeks. However, the antibiotic concentration was kept constant, whereas the typical release from monolithic device gives an initial peak and then diminished levels. If the proposed drug delivery device still achieves high enough drug levels (say 1000 times the minimum inhibitory concentration of the inoculum) for a critical number of days, then biofilm eradication would be possible. But if the antibiotic level was too low, then the device may fail to eradicate biofilms.

Does biofilm treatment require high antibiotic level for the full treatment duration? Our experiments in the preceding chapter investigated a constant antibiotic level as they focused on identifying what the minimum biofilm eradication concentration and treatment duration were. However, biofilm eradication might not require the high antibiotic level throughout, and perhaps an initial high level followed by several weeks' treatment with a lower level might be effective. Therefore, any PLGA monolithic device that gives a high initial antibiotic level and a lower longer term level might still be effective against biofilms, with the initial burst release being an advantage even though zero-order kinetics is not achieved. However, the post-peak level still needs to be higher than the level needed to eradicate biofilms or planktonic bacteria, otherwise these lower antibiotic levels may be in the range that encourages survival of emerging resistant mutants.

In order to obtain a more constant release from monolithic systems a number of approaches have been suggested (Anderson and Kim 1984). Diffusion of the drug may be delayed by reducing the ingress of water, and this could be

achieved by strategies that control the rate of swelling, or by having an internal aqueous boundary layer that provides resistance to drug release (ideally, erosion should be confined to the outer surface of the polymer, so that drug is released only from the outside first with the release zone gradually moving to the centre of the device). The addition of an impervious surface barrier has also been suggested (Anderson and Kim 1984), and the amorphous melted edges seen in our device (Fig 10.3.2.3) are therefore not necessarily a disadvantage. The sintering process, making objects from powders (in our case by heating microparticles in powder form to make a shaped pellet), results in PLGA particles adhering to each other because heating them above the glass transition temperature of PLGA (45 – 50°C) changes them from being hard into a molten rubber-like state during which particles adhere. Although the majority of the pellet contained a latticework of individual PLGA spheres adherent to each other at their edges with pores in between, the edges of the pellets had a fused molten appearance, where the particles turned into a solid rather than a porous network seen in the rest of the pellet. This presumably occurred because the temperature was higher at the edges than in the centre of the pellet and could be due to heat transfer from the mould. This solid surface layer would not necessarily be a disadvantage, because it would delay water ingress into the device and therefore potentially delay drug release.

Hydrogels

In addition to a PLGA matrix, the device used in these studies also incorporated a hydrogel (carboxymethylcellulose or poloxamer) to improve

drug release properties. As discussed above, the PLGA-based monolithic formulations have distinct advantages, but there is a tendency for very water-soluble drugs to diffuse from the matrix quickly because both erosion and diffusion control drug release. Therefore, to prolong drug release that would be possible with a simple PLGA device, a hydrogel was incorporated into the device.

Hydrogels have been widely studied for their modified release properties (Anderson and Kim 1984). They are polymeric materials consisting of hydrophilic macromolecules cross-linked to form a three-dimensional network, and have the ability to imbibe large quantities of water without dissolving (Simoes, Figueiras and Veiga 2012). Drug molecules can then be dispersed or dissolved within the hydrogel and its retained water, and are released through diffusion. Release is influenced by geometry of the device, type of hydrogel and degree of cross-linking, as well as water content of the hydrogel and solubility of the drug. For example, increasing hydration leads to increased diffusion (Yasuda, Lamaze and Ikenberry 1968), whilst increased cross-linking leads to reduced pore size, less swelling, and lower diffusion (Reinhart, Korsmeyer and Peppas 1981).

Hydrogels resemble natural tissues more than any other synthetic biomaterial, and have excellent biocompatibility. However, their structural integrity is limited so that they have a tendency to move away from the site of application unless constrained, loading of hydrophobic drugs may be limited, and their

high water content and large pore size may lead to relatively rapid drug release (Simoes, Figueiras and Veiga 2012).

Carboxymethylcellulose

CMC (Fig 2.5.3.3) is a cellulose derivative that is widely used as a food viscosity modifier, is a component of many tooth pastes, forms the outer gel lining of nasal packing (Arthrocare 2011) or wound dressings (Lim, et al. 2010), and also has modified release application. It has been used to disperse modified release tablets (Ishino, et al. 1992), as a modified release gel (Barbucci, Leone and Vecchiullo 2004), to form a biodegradable membrane used in drug release (Jain, Carvalho and Banerjee 2010), and to encapsulate antibiotics (Ertan, et al. 1997).

CMC is available in a range of viscosities (Hoefler 2011). Viscosity is governed by the chain length of the CMC (degree of polymerisation), and the higher the degree of polymerisation the greater the molecular weight and the more viscous the solution; the high viscosity CMC produced by Aqualon from naturally occurring fine cotton fibres (cellulose) has a degree of polymerisation of 3,200 with a molecular weight of 700,000 (Aqualon 2000). High viscosity CMC produces a thick viscous mixture rather than a thinner liquid seen with low viscosity CMC, although the concentration used also affects the viscosity of the final solution as does temperature, pH and any chemical modifications. Higher viscosity leads to a reduced drug release rate (Emeje, Kunle and Ofoefule 2006) (Hiremath and Saha 2008) and was therefore chosen for these experiments.

CMC is widely used and generally considered safe, although a few cases of allergy have been reported (Dumond, et al. 2009). It has been investigated as middle ear packing material (Shen, et al. 2011), with one animal study finding no concerns regarding hearing (Jang, et al. 2008). However, another found a deterioration in hearing, although this later study is open to criticism. The researchers found elevated hearing thresholds (Antonelli, Sampson and Lang 2010), and assumed this was due to damage to the inner ear on the basis that the middle ear appeared healthy; however, the inner ear was not specifically examined, and the animals experienced no balance problems despite the fact that damage to the inner ear often affects both hearing and balance. The presence of packing material might then have interfered with sound transduction through the middle ear, thus causing a conductive loss rather than inner ear injury.

Poloxamer

Poloxamers (Fig 2.5.3.2), also known by their trade name pluronics, are triblock co-polymers, consisting of a central hydrophobic polyoxypropylene chain flanked by two hydrophilic polyoxyethylene chains. This amphiphilic structure gives poloxamers surfactant properties, and they can be used to stabilise cell membranes and prevent apoptosis (Medina, et al. 2011). They have also been used to overcome multidrug-resistance in cancer cells (Zhang, et al. 2010), and have immune modulatory properties (for example increasing antibody production and enhancing macrophage function) (Moghimi and Murray 1996), in addition to being used in modified drug release (Chae, Mo

and Oh 2010) (Desai and Blanchard 1998) (Manjunath, Venkateswarlu and Hussain 2011). They are derived from natural oil and gas.

All poloxamers are composed of the same monomers, but variation in the length of the polymer chain and exact percentage polyoxyethylene content lead to variation in the molecular weight, physical form, and drug release properties. Pluronic F127 (also known as poloxamer 407) contains polyoxypropylene with an approximate molecular weight of 3600, and is approximately 70% polyoxyethylene. A 20% solution is liquid at temperature below 15°C but turns to gel at 25 to 60°C (Escobar-Chávez, et al. 2006) (Ohta, et al. 2006). These thermo-reversible properties make it attractive for drug delivery as administration of a liquid is easier, but a gel remains localised (Wang, Dellamary, et al. 2009) (Wang, Dellamary, et al. 2011). Pluronic F127 also has muco - adhesive properties, another characteristic useful to keep the gel in place (Dumortier, et al. 2006).

Poloxamers are widely used in cosmetics and mouthwashes, and generally considered safe (Singh-Joy and McLain 2008). Guinea pig studies have investigated pluronic F127 as an agent to deliver drugs to the middle and inner ear, with no ototoxicity found. Although two teams of investigators both noted temporary deterioration in hearing, the fact that this reverted to normal suggested that the hearing loss was caused by the physical presence of the gel (conductive loss), rather than an injury to the inner ear. One group noted a transient hearing change that reversed within a week (Wang, Dellamary, et al. 2009) (Wang, Dellamary, et al. 2011), whilst the other group found temporary

hearing loss that recovered by the end of the study at 49 days (H. Feng, et al. 2007) (Feng, Sun and Jiang 2008), with no histological abnormality of the middle ear or the cochlea seen. The latter study also concluded that practically all the gel had disappeared 49 days after application.

Degradation of drug delivery device

PLGA undergoes hydrolysis to lactic and glycolic acid, both of which are metabolised in man without adverse reaction. CMC can be degraded by cellulase, but this enzyme is not present in man (Reeves, et al. 2010); some bacteria can metabolise cellulose, but the bacteria commonly involved in OME do not (Schwarz 2012).

Poloxamers are also not biodegradable, and are rapidly excreted in the urine following systemic administration (Singh-Joy and McLain 2008). Both CMC and Poloxamer may therefore be expected to be absorbed or dissipated down the Eustachian tube once the PLGA device degrades. The behaviour of PLGA and Poloxamer or CMC device *in vivo* would have to be examined prior to clinical use, to ensure appropriate degradation and avoid any toxicity.

10.4.6 Sterilisation

The sterility assessment shows that the pellets were sufficiently sterile for the purposes of these studies. However, only limited sterilisation tests were carried out, and any future clinical work would require a more detailed sterilisation assessment. As the sterility assessment in these studies relied on detection of bacteria on sheep blood agar, any contaminant microorganisms

that do not grow on sheep blood agar would not be detected. Whilst the presence of any such undetected organisms would be unlikely to interfere with results of experiments on eradication (where only blood agar was used), pellets tested in this way would clearly not be adequate for clinical use. It is possible that the pellets may have contained bacteria not detected by our experiments and, although unlikely, such bacteria may have affected the modified release properties.

Sterility testing is a complex process (Kowalski 2009), guided by official legislation such as the United States Pharmacopeia (Tirumalai 2009) and the British Standards Institute (Wickhamlabs 2011). Any medicinal products / devices are required to pass strict tests, such as immersion of the device in a nutrient medium for 14 days to see if any bacteria grow, direct inoculation, or the Millipore Steritest system that allows direct sampling of medical devices with minimised contamination risk (Millipore 2011). Additional tests include confirmation of absence of bacteriostatic or fungistatic agents, by inoculating the device with set pre-determined micro-organisms and ensuring that they do grow.

The biodegradable polymers used in drug delivery, such as PLGA, pose particular problems when it comes to sterilisation because they are temperature-sensitive, and therefore heat-based methods would not be appropriate. They are also generally susceptible to degradation or alteration by sterilisation processes, although this does not necessarily affect drug release.

Ultraviolet (UV) light was used to sterilise the pellet components in these experiments. Ultraviolet germicidal irradiation (UVGI) uses UV light between 200 and 320 nm to break the molecular bonds within microorganisms, leading to DNA damage so that the microorganism can no longer reproduce (Kowalski 2009). UV achieves good surface disinfection, but it does not penetrate well. Therefore, the pellet components were sterilised prior to being mixed and solidified, because the components spread thinly as a powder or gel will be more accessible to surface sterilisation. The second exposure to UV once the pellets were made then again sterilised the surface of the actual pellet, the surface being the most likely area to have been contaminated when removing the pellets from the mould.

UV light has been previously used to sterilise a PLGA-based drug delivery system, but it may affect drug release (for example attenuating release of encapsulated growth factors) (Moioli, et al. 2006), and it can also damage the PLGA scaffold itself (Shearer, et al. 2006), thus affecting release patterns and device life. Gamma irradiation has also been widely used to sterilise PLGA (Li and Jasti 2006), and appears to have no (Igartua, et al. 2008) or little (Jain, et al. 2011) (Lee, et al. 2003) effect on drug release, although it may affect polymer properties (Dorati, et al. 2008) (Friess and Schlapp 2006). Similarly, electron-beam radiation has been widely used, and it is known to lead to a decrease in the molecular weight of PLGA through chain scission (Loo, OOi and Boey 2005). Ethylene oxide has also been used (Pietrzak 2010) but may have a greater effect on drug release, for example causing particle

aggregation and greater initial drug release (Friess and Schlapp 2006). Is also likely that different formulations are affected in different ways by the sterilisation process.

The sterilisation process will also need to ensure that the active ingredients are not affected. For example, rifampicin is known to be light sensitive, and it is possible that it may be affected by UV sterilisation, although a literature search failed to find any research on the topic. Clearly rifampicin was active in the research presented here, but patient use would require avoidance of processes that could affect antibiotic potency.

Whilst UV sterilisation was satisfactory for these laboratory experiments, any future clinical work would require detailed sterility assessment. This should include not only tests of sterility, but should also test the effect of sterilisation modality on the particular modified release formulation chosen, and should ensure that the sterilisation process does not have any residual effects that would be toxic to man.

10.5 SUMMARY: ANTIBIOTIC PELLETS

Appropriately sized microparticles of PLGA can be prepared using the emulsion method, and antibiotic pellets made with the aid of a PTFE and stainless steel mould. These pellets will be used in the following chapters that examine drug release from the pellets and biofilm eradication.

The monolithic PLGA matrix device combined with a hydrogel (poloxamer or carboxymethylcellulose) used in these experiments has a number of advantages, especially its relative simplicity, ability to vary the size of the device and amount of antibiotics, and the relative safety of the agents chosen; it is thus an ideal starting point when exploring the possibility of a modified-release preparation for the treatment of OME biofilms. However, such devices do have a number of possible disadvantages, chiefly the tendency to release a large amount of drug in the initial stages with lower levels later on, and this may or may not influence the ability of the device to eradicate biofilms. Prior to any clinical studies, the particle and pellet manufacture and sterilisation will require improvement, with toxicity studies also warranted to ensure patient safety.

11 ANTIBIOTIC PELLETS:

DRUG RELEASE AND ANTI-BIOFILM ACTIVITY

11.1 INTRODUCTION

The understanding that biofilms play an important role in the pathogenesis of OME (Chapter 7) (Hall-Stoodley, et al. 2006) opens up the potential for better new treatments that could decrease the high recurrence rate after grommet extrusion (Chapter 5) (Gates, Avery and Prihoda 1987) (Maw and Bawden 1994) (Rosenfeldt and Bluestone 1999). On the basis of previous work demonstrating that eradication of biofilms with high dose antibiotics is possible (Chapter 9) (Ceri, et al. 1999) (Donlan and Costerton 2002) (Nickel, et al. 1985) (Olson, et al. 2002) (Vorachit, et al. 1993), we set out to develop and test a novel biodegradable modified-release antibiotic pellet as a potential anti-biofilm treatment for OME.

This chapter shows the determination of the amount, pattern and duration of drug release from the antibiotic pellets. We had chosen to investigate a pellet based on PLGA (56 kDa, 50:50, mean particle size 12 μm) with rifampicin and clindamycin, combined with a carrier hydrogel, either pluronic F128 or carboxymethylcellulose (CMC). The antibiotic concentration was varied, with three different levels chosen (called low, medium and high); the amount of clindamycin loaded into the pellet was higher than rifampicin, due to the different susceptibility of the *S aureus* test organism. The main comparisons

included the different antibiotic levels, and a comparison between pluronic (20%) and high viscosity CMC (2%). Additional comparisons included medium viscosity CMC (6%), shortened sintering time (1 or 4, instead of 16 hours), and a change in the ratio of PLGA to carrier (from 1:0.8 to 1:0.6).

The composition of the different pellets studied is shown in Table 11.1.1. Inactive pellets containing no antibiotic were used as controls.

NAME	Carrier	PLGA:carrier ratio	Sintering time	Rifampicin	Clindamycin
CMC/L	CMC (hv)	1:0.8	16 hrs	0.5%	3%
CMC/M	CMC (hv)	1:0.8	16 hrs	1%	6%
CMC/H	CMC (hv)	1:0.8	16 hrs	4%	24%
CMC/L(mv)	CMC (mv)	1:0.8	16 hrs	0.5%	3%
CMC/L(1:0.6)	CMC (hv)	1:0.6	16 hrs	0.5%	3%
CMC/L(1h)	CMC (hv)	1:0.8	1 hr	0.5%	3%
CMC/L(4h)	CMC (hv)	1:0.8	4 hrs	0.5%	3%
PL/L	pluronic	1:0.8	16 hrs	0.5%	3%
PL/M	pluronic	1:0.8	16 hrs	1%	6%
PL/H	pluronic	1:0.8	16 hrs	4%	24%

Table 11.1.1: Composition of the pellets studied. Antibiotic percentage expressed as a % of PLGA weight. hv: high viscosity. mv: medium viscosity

Pellet effectiveness was assessed in three ways. High Performance Liquid Chromatography (HPLC) was used as a quantitative method that gives details

on the exact amount released. We also used Serial Plate Transfer Testing (SPTT), where the antibiotic pellet is transferred to a freshly inoculated agar plate every day and the zone of bacterial inhibition around the pellet measured (Bayston, Grove, et al. 1989) (Furno, et al. 2004); the zone of inhibition is related to the amount of drug released and thus SPTT is also a quantitative method, and although it does not give an exact dose of drug released it demonstrates the inhibitory behaviour of the pellets against actual bacteria. For HPLC testing the pellet was immersed in phosphate-buffered saline (PBS) and the PBS tested to determine amount of drug released, but in SPTT the pellet sits on top of an agar plate with one surface involved in release, and antibiotics diffusing out of pellet into the agar to inhibit bacterial growth. Thus the test conditions in HPLC and SPTT are different. Lastly, the pellet effectiveness was examined against *S aureus* biofilms in the *in vitro* model developed in this thesis (Chapter 8).

11.2 METHODS

11.2.1 High-performance liquid chromatography

A new HPLC method was developed for our purposes; this section describes the final method adopted, with the results section in this chapter providing more details. In order to study the release of drugs from the pellets using HPLC, the pellets were placed into 1 mL of PBS in an Eppendorf tube. Every 2 days the PBS was removed, the pellet weighed, and fresh PBS replaced; the solution containing released antibiotics was stored at -20°C prior to analysis.

HPLC was performed with Beckman System Gold (Beckman Coulter, High Wycombe, UK), using the Eclipse XDB-C8 (Agilent Technologies, Stockport, UK) HPLC column (150 mm long, 4.6 mm internal diameter, 5 μm sized silica particles with an 8 carbon side chain), with a flow rate of 1mL/min at 40°C. The mobile phase consisted of aqueous sodium dihydrogen phosphate (15mM, pH2.5) with 10% acetonitrile, mixed with a varying amount of methanol (65 to 80%) in a gradient elution method as shown in Fig 11.2.1.1. Detection was performed at 254 nm for rifampicin and 210 nm for clindamycin.

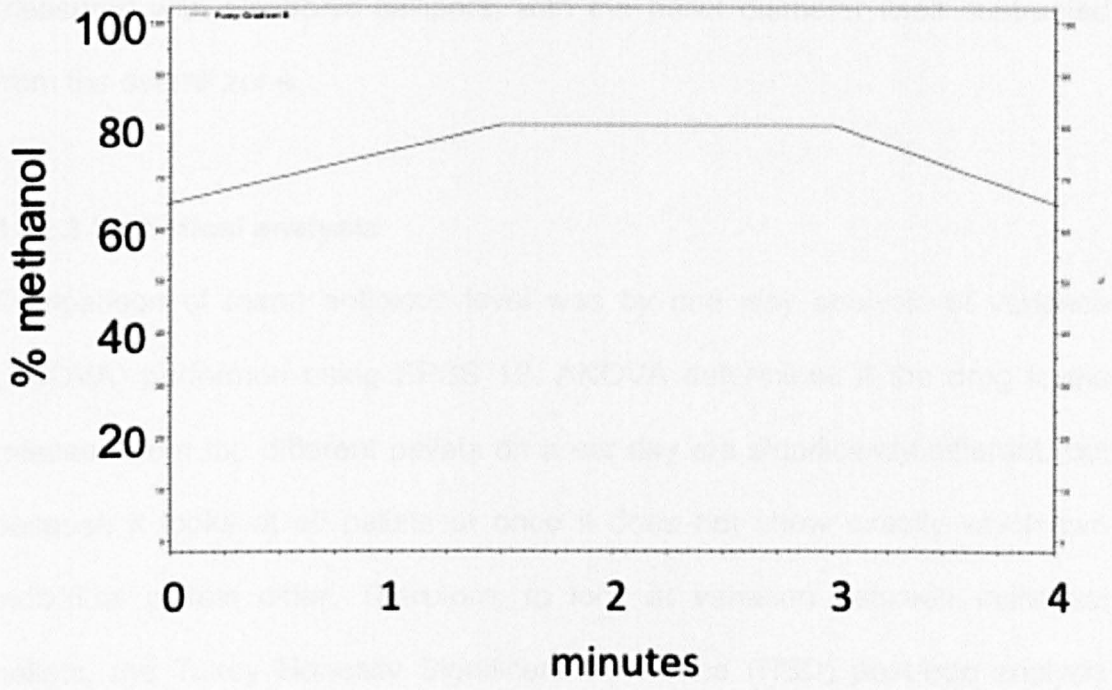


Fig 11.2.1.1: HPLC gradient elution method showing how the percentage of methanol varies with time.

11.2.2 Serial plate transfer test

The release of antibiotics was assessed with serial plate transfer test (SPTT). This involves the transfer of the antibiotic device to a freshly inoculated agar plate to assess duration of release, with the size of the zone of inhibition giving quantitative information about the amount of antibiotic released at defined time periods. A suspension of *S aureus* (F2315) was prepared to an optical density of 0.08-0.13 (McFarland 0.5 equivalent) at 490 nm measured using Jenway Multicell Changer Spectrophotometer (Bibby Scientific Limited, Stone UK), and 200 µL used to inoculate an Iso-Sensitest agar plate. The diameter of the zone of inhibition of bacterial growth around the pellet was

measured with electronic callipers, with the pellet diameter itself subtracted from the overall zone.

11.2.3 Statistical analysis

Comparison of mean antibiotic level was by one way analysis of variance (ANOVA) performed using SPSS 19. ANOVA determines if the drug levels released from the different pellets on a set day are significantly different, but because it looks at all pellets at once it does not show exactly which two individual pellets differ. Therefore, to look at variation between individual pellets, the Tukey Honestly Significant Difference (HSD) post-hoc analysis was performed (Stevens 1999). The advantage of analysing data this way, i.e. ANOVA on all pellets followed by post-hoc comparisons of two pellets at a time, is that the Tukey HSD method corrects for the fact that multiple comparisons are being made; if individual pellets were compared to each other using the Student's t test then a large number of comparisons is being made, and statistically 5% of those comparisons will erroneously turn out to be "statistically significant" just by chance.

11.3 RESULTS

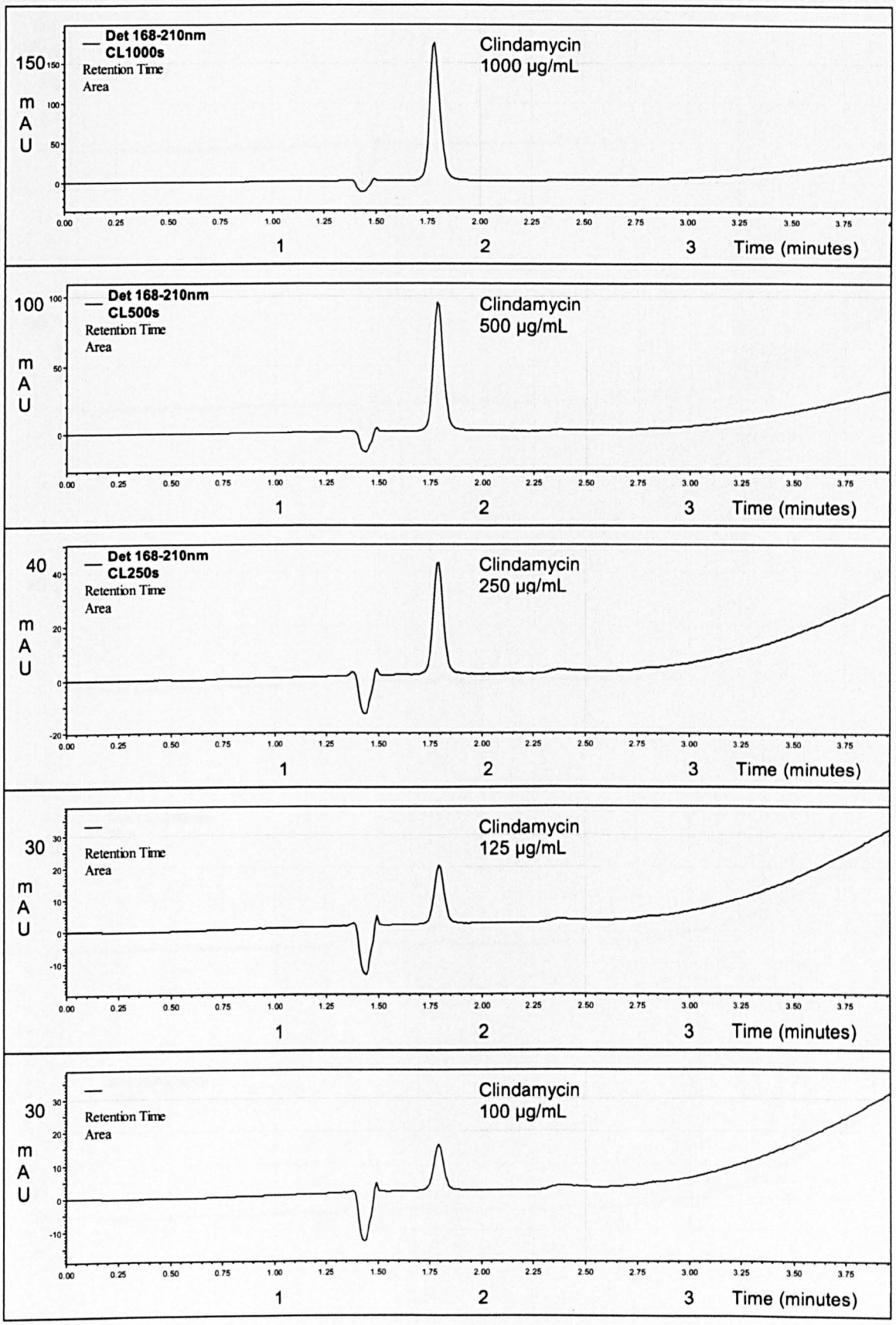
11.3.1 High performance liquid chromatography method

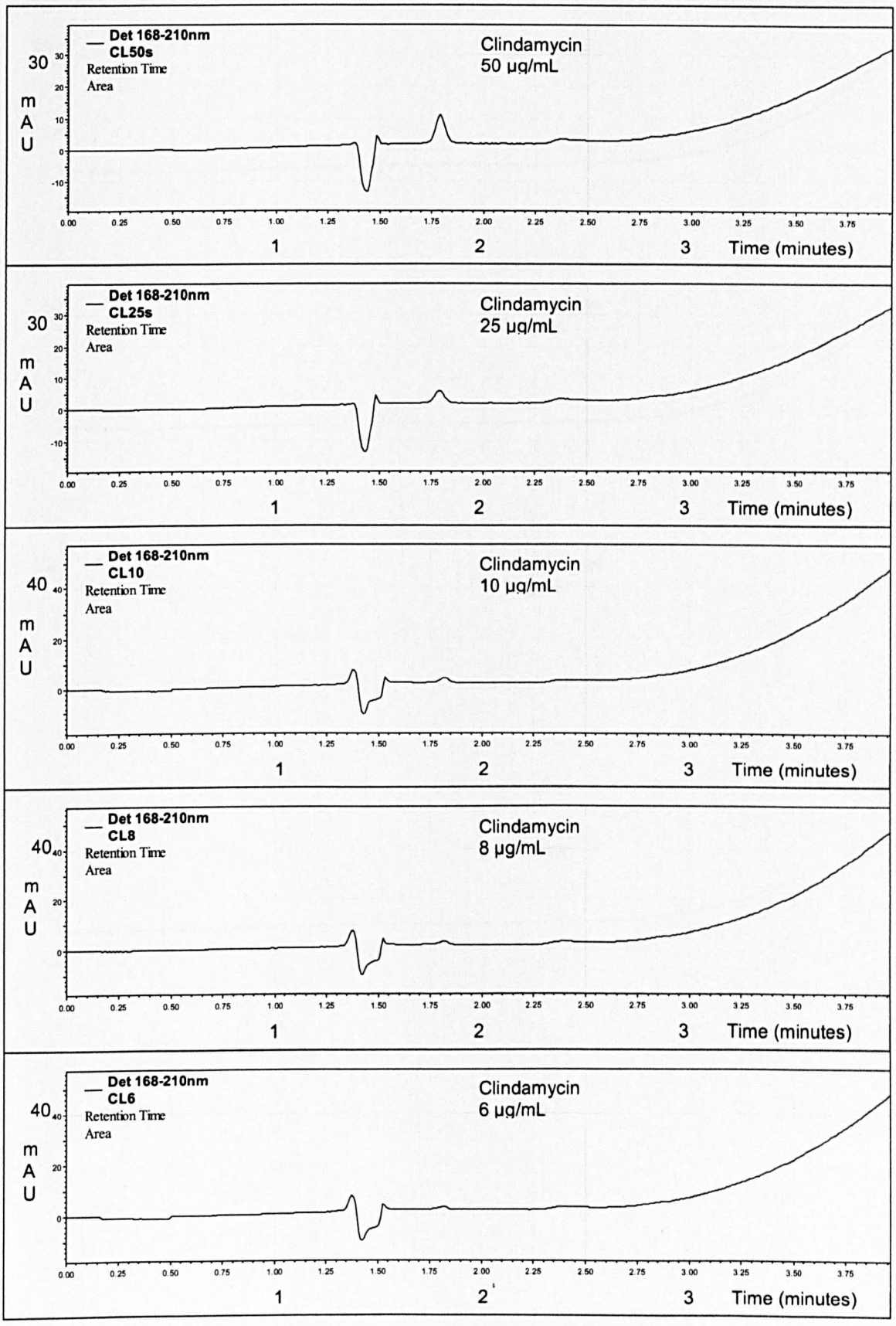
A new HPLC method to simultaneously detect rifampicin and clindamycin was developed. In HPLC, each substance has a typical retention time for a given method, with the substance identified using an ultraviolet (UV) detector. The HPLC output is in the form of a chromatogram (Fig 11.3.1.1), plotting time on the x axis and light absorption at the specified wavelength on the y axis (the scale of the y axis is adjusted depending on peak size). Each substance thus shows up as a peak at its retention time, around 1.8 minutes for clindamycin, and just over 3.0 minutes for rifampicin with the HPLC method employed. The chromatograms also contain a baseline deflection just before 1.5 minutes, representing the time when the solvent front moving through the column reaches the UV detector; this is not seen on all the chromatograms because the solvent front deflection is small and seen only on those chromatograms with a small antibiotic levels and thus a small y axis scale.

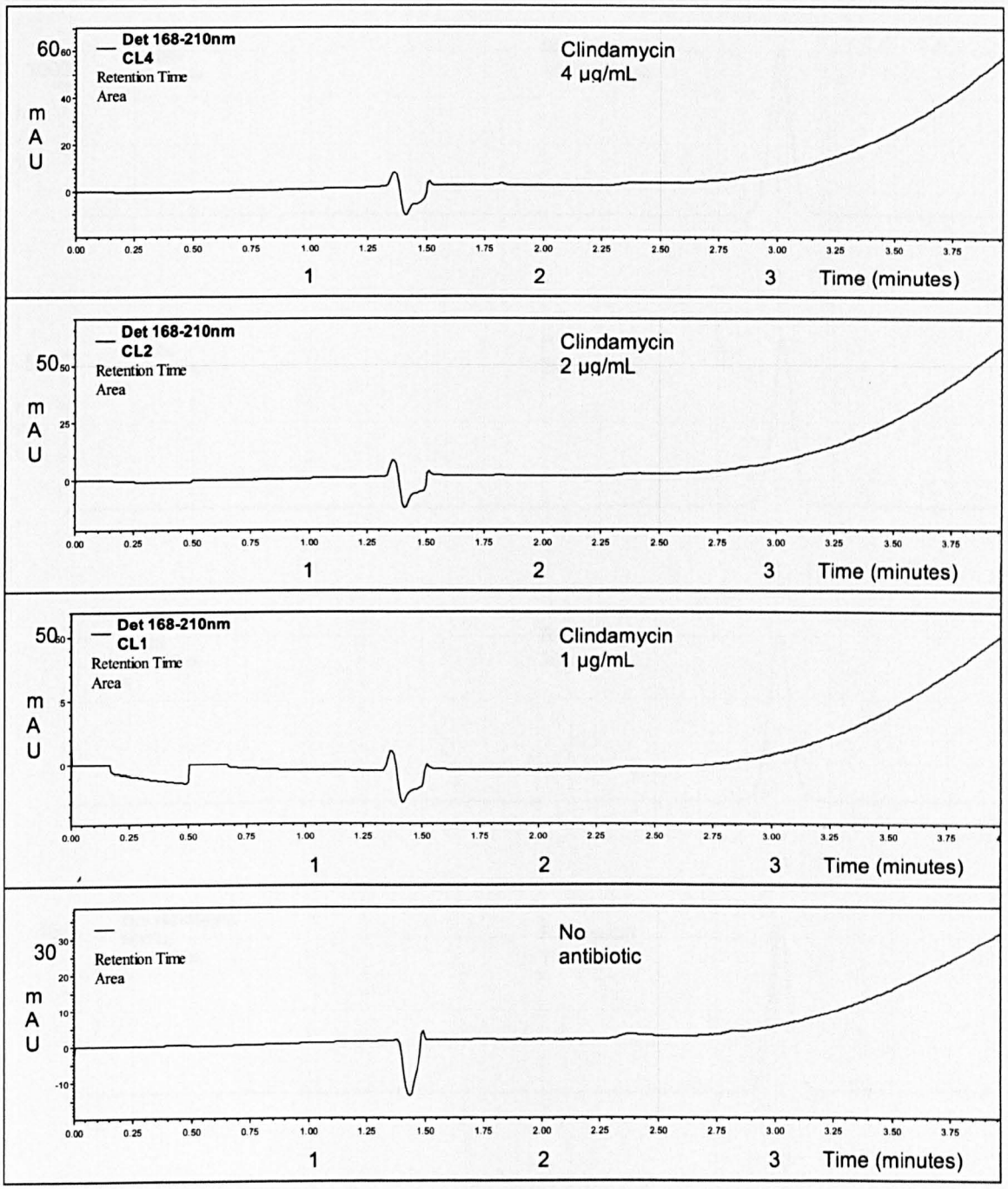
An ultraviolet absorption scan of rifampicin and clindamycin showed that the best absorption wavelength for the former was 254 nm, and the latter 210 nm. Rifampicin was also seen at 210 nm, although not as well as at 254 nm. Clindamycin was seen only at 210 nm; although some chromatograms traces at 254 nm did show a deflection between 1.75 and 2 minutes, this was not due to clindamycin (because it was also seen on controls that contained no clindamycin) but reflected baseline variation, unlike peaks at 210 nm that were genuinely clindamycin.

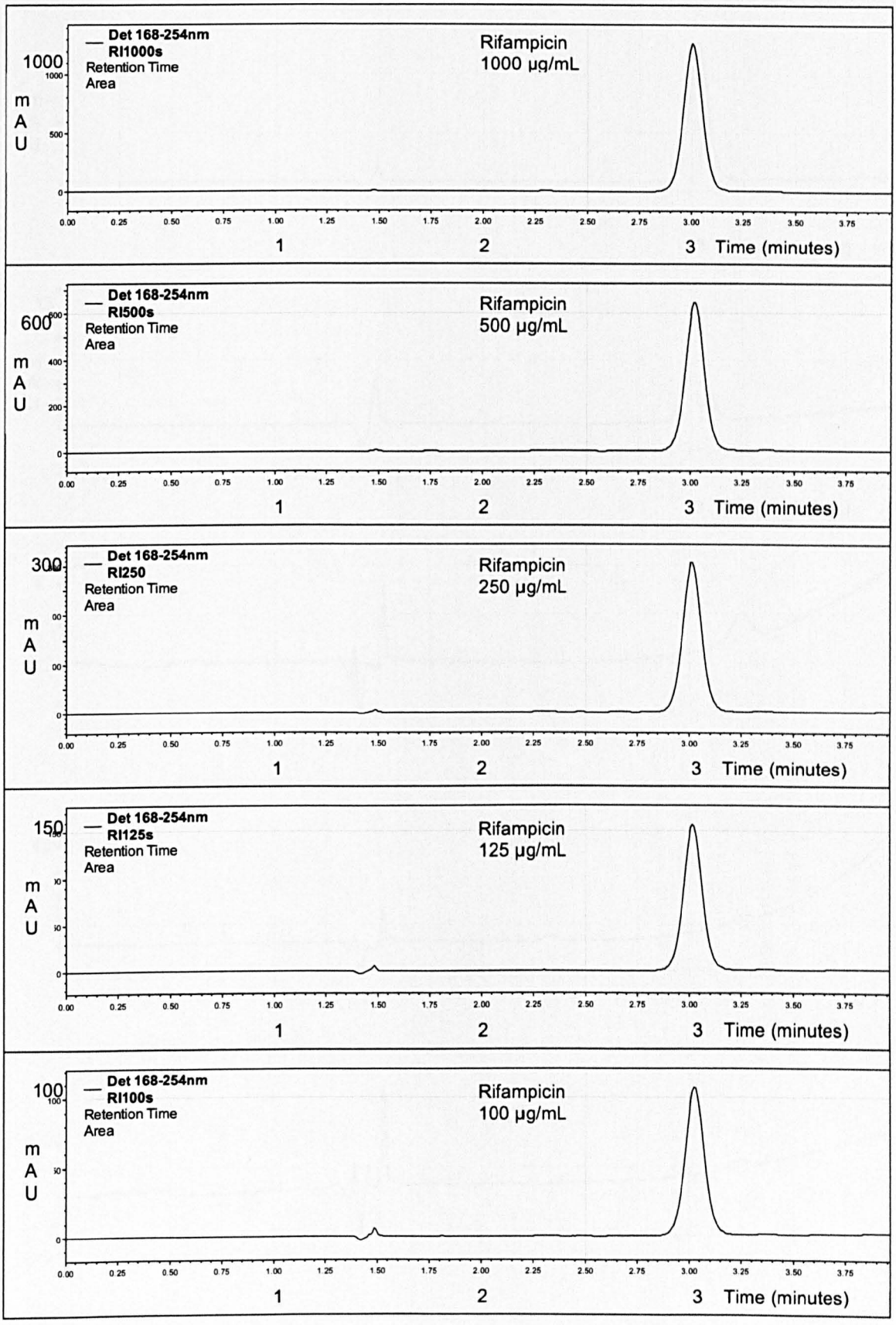
The chromatograms (Fig 11.3.1.1) showed that both antibiotics could still be detected down to 2 µg/mL.

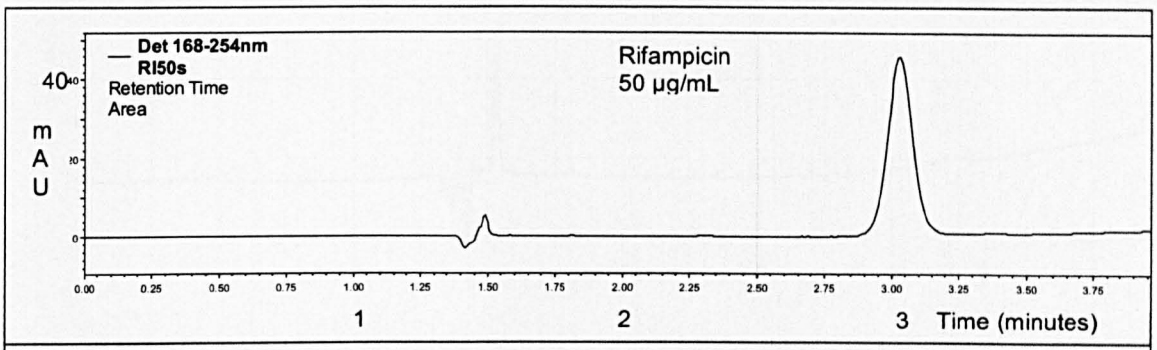
Fig 11.3.1.1 (following pages): HPLC chromatograms with clindamycin (210 nm) and rifampicin (254 nm) standards (indicated in boxes on each chromatogram). The x axis shows the retention time; the y axis (mAU, milli absorption units) scale is adjusted depending on the size of the peak.

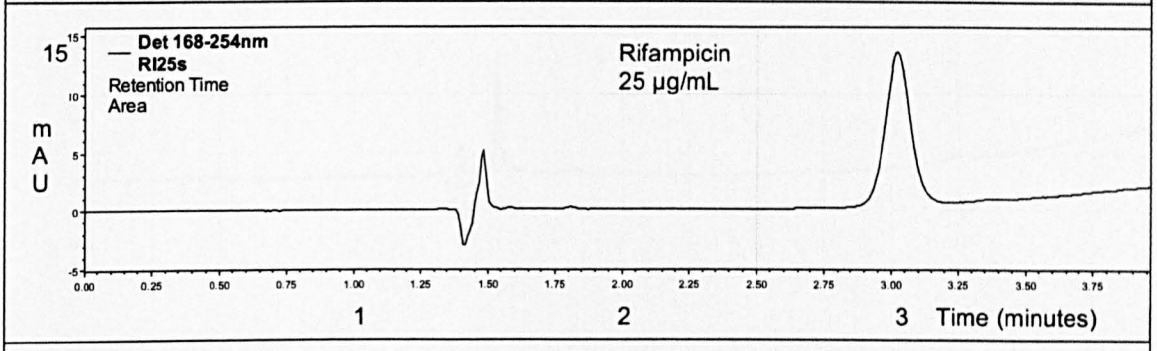

Det 168-210nm
CL1000s
Retention Time
Area
Clindamycin
1000 µg/mL
150
m
A
U
1
2
3
Time (minutes)
Det 168-210nm
CL500s
Retention Time
Area
Clindamycin
500 µg/mL
100
m
A
U
1
2
3
Time (minutes)
Det 168-210nm
CL250s
Retention Time
Area
Clindamycin
250 µg/mL
40
m
A
U
1
2
3
Time (minutes)
Retention Time
Area
Clindamycin
125 µg/mL
30
m
A
U
1
2
3
Time (minutes)
Retention Time
Area
Clindamycin
100 µg/mL
30
m
A
U
1
2
3
Time (minutes)

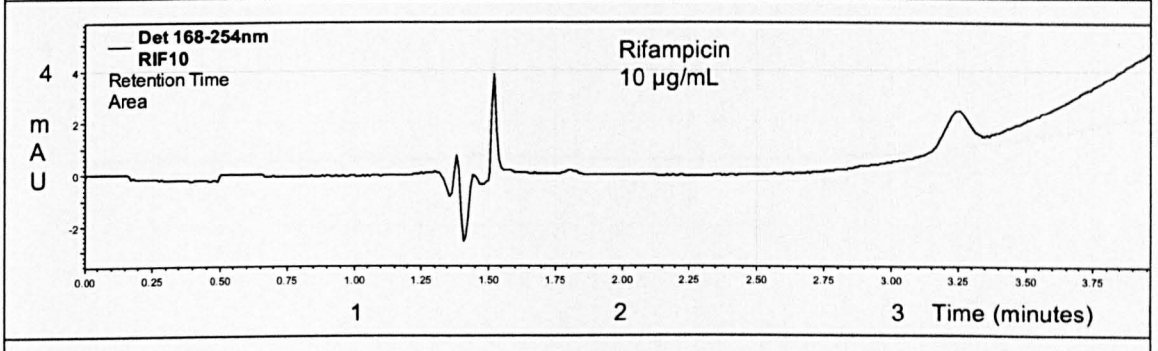

Det 168-210nm
CL50s
Retention Time
Area
Clindamycin
50 µg/mL
mAU
Time (minutes)
Det 168-210nm
CL25s
Retention Time
Area
Clindamycin
25 µg/mL
mAU
Time (minutes)
Det 168-210nm
CL10
Retention Time
Area
Clindamycin
10 µg/mL
mAU
Time (minutes)
Det 168-210nm
CL8
Retention Time
Area
Clindamycin
8 µg/mL
mAU
Time (minutes)
Det 168-210nm
CL6
Retention Time
Area
Clindamycin
6 µg/mL
mAU
Time (minutes)

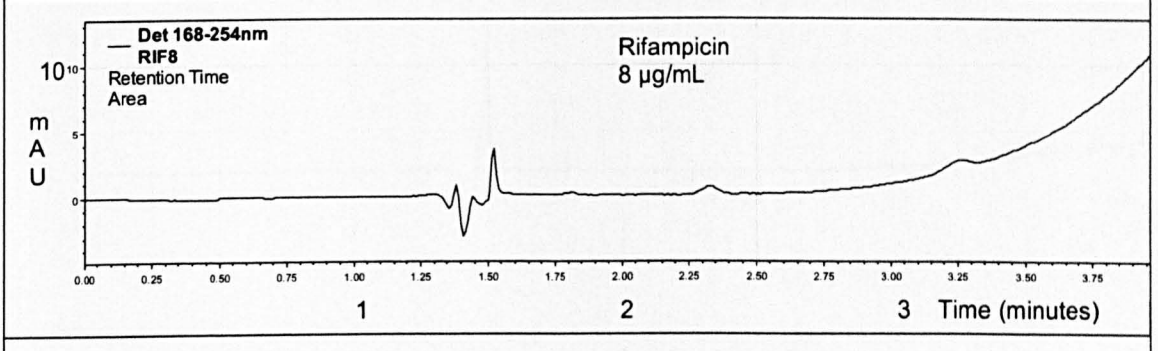

Det 168-210nm
CL4
Retention Time
Area
Clindamycin
4 µg/mL
mAU
Time (minutes)
Det 168-210nm
CL2
Retention Time
Area
Clindamycin
2 µg/mL
mAU
Time (minutes)
Det 168-210nm
CL1
Retention Time
Area
Clindamycin
1 µg/mL
mAU
Time (minutes)
Retention Time
Area
No
antibiotic
mAU
Time (minutes)

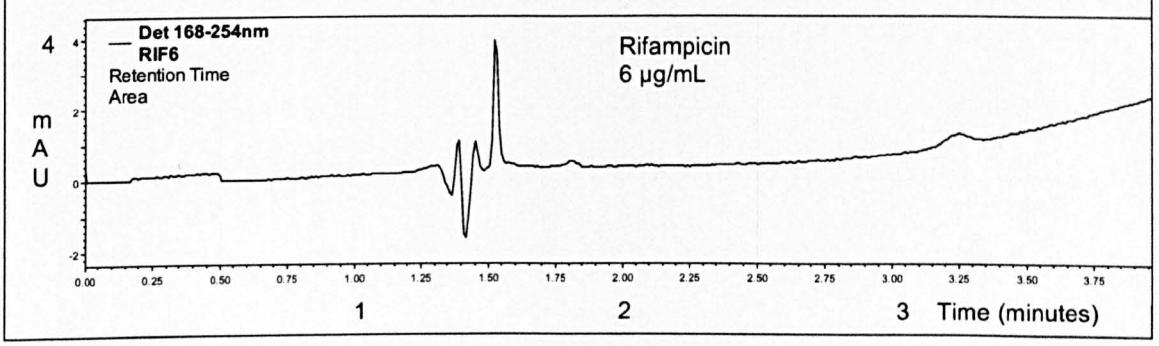

Det 168-254nm
RI1000s
Retention Time
Area
Rifampicin
1000 µg/mL
mAU
1000
500
0
0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00 2.25 2.50 2.75 3.00 3.25 3.50 3.75
1 2 3 Time (minutes)
Det 168-254nm
RI500s
Retention Time
Area
Rifampicin
500 µg/mL
mAU
600
400
200
0
0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00 2.25 2.50 2.75 3.00 3.25 3.50 3.75
1 2 3 Time (minutes)
Det 168-254nm
RI250
Retention Time
Area
Rifampicin
250 µg/mL
mAU
0
0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00 2.25 2.50 2.75 3.00 3.25 3.50 3.75
1 2 3 Time (minutes)
Det 168-254nm
RI125s
Retention Time
Area
Rifampicin
125 µg/mL
mAU
0
0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00 2.25 2.50 2.75 3.00 3.25 3.50 3.75
1 2 3 Time (minutes)
Det 168-254nm
RI100s
Retention Time
Area
Rifampicin
100 µg/mL
mAU
100
0
0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00 2.25 2.50 2.75 3.00 3.25 3.50 3.75
1 2 3 Time (minutes)

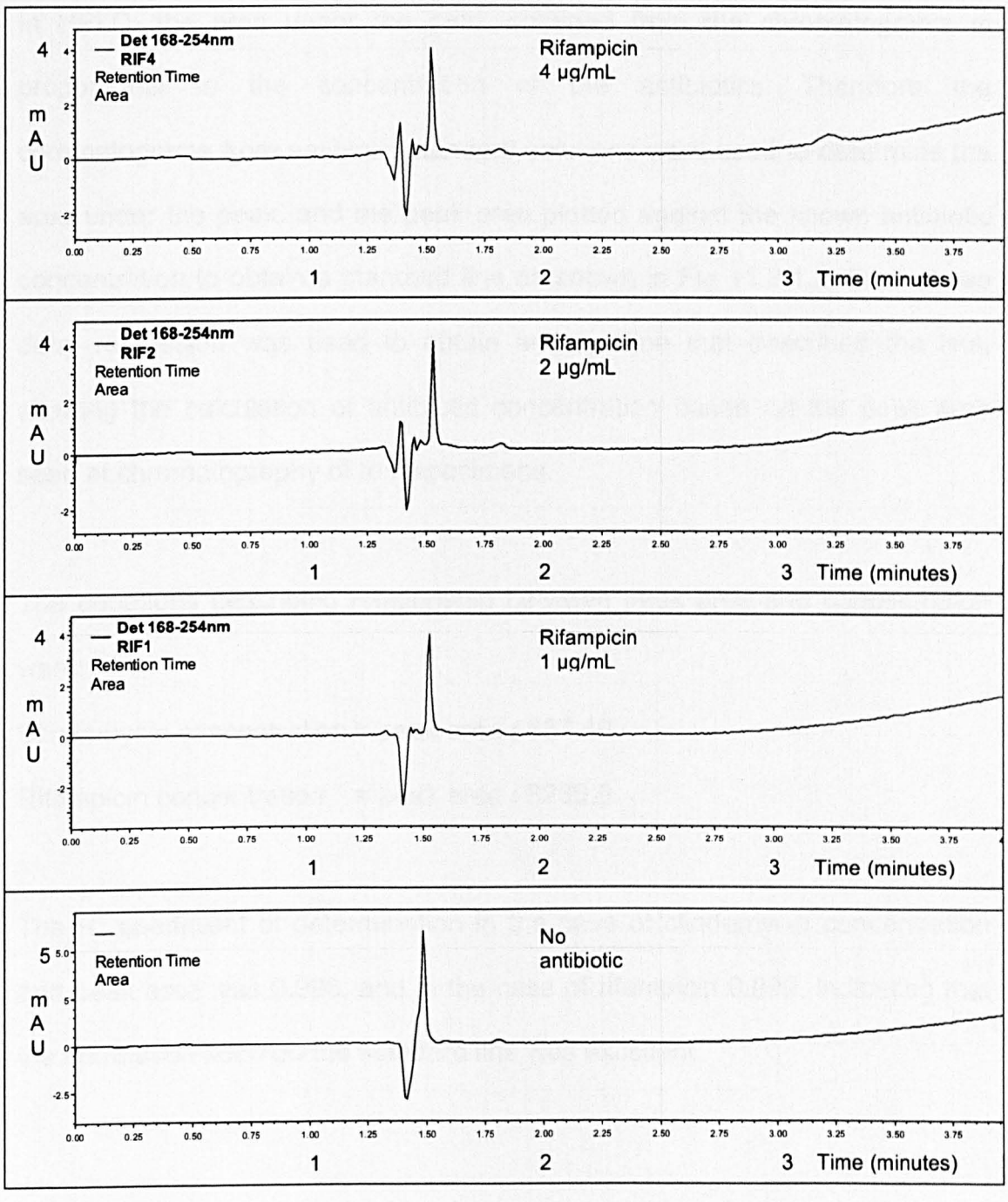

Det 168-254nm
RI50s
Retention Time
Area
Rifampicin
50 µg/mL
mAU
1
2
3
Time (minutes)


Det 168-254nm
RI25s
Retention Time
Area
Rifampicin
25 µg/mL
mAU
1
2
3
Time (minutes)


Det 168-254nm
RIF10
Retention Time
Area
Rifampicin
10 µg/mL
mAU
1
2
3
Time (minutes)


Det 168-254nm
RIF8
Retention Time
Area
Rifampicin
8 µg/mL
mAU
1
2
3
Time (minutes)


Det 168-254nm
RIF6
Retention Time
Area
Rifampicin
6 µg/mL
mAU
1
2
3
Time (minutes)


Det 168-254nm
RIF4
Retention Time
Area
Rifampicin
4 µg/mL
mAU
Time (minutes)
Det 168-254nm
RIF2
Retention Time
Area
Rifampicin
2 µg/mL
mAU
Time (minutes)
Det 168-254nm
RIF1
Retention Time
Area
Rifampicin
1 µg/mL
mAU
Time (minutes)
Retention Time
Area
No
antibiotic
mAU
Time (minutes)

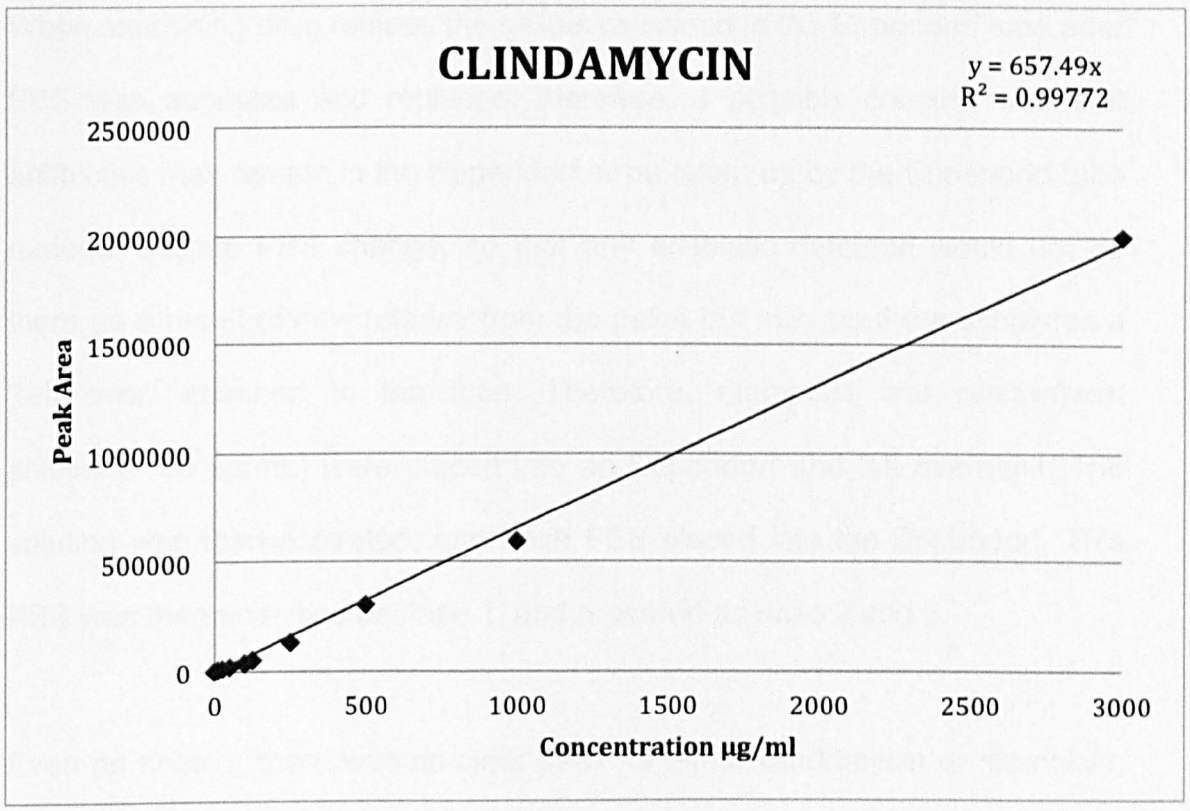
In HPLC, the area under the peak obtained from the chromatograms is proportional to the concentration of the antibiotics. Therefore the chromatograms from antibiotic standard solutions were used to determine the area under the peak, and the peak area plotted against the known antibiotic concentration to obtain a standard line as shown in Fig 11.3.1.2. From these data, regression was used to obtain an equation that described the line, allowing the calculation of antibiotic concentration based on the peak area seen at chromatography of test specimens.

The equations describing relationship between peak area and concentration were:

Clindamycin concentration = peak area / 657.49

Rifampicin concentration = peak area / 8236.9

The R^2 coefficient of determination in the case of clindamycin concentration and peak area was 0.998, and in the case of rifampicin 0.999, indicating that the correlation seen on the standard line was excellent.



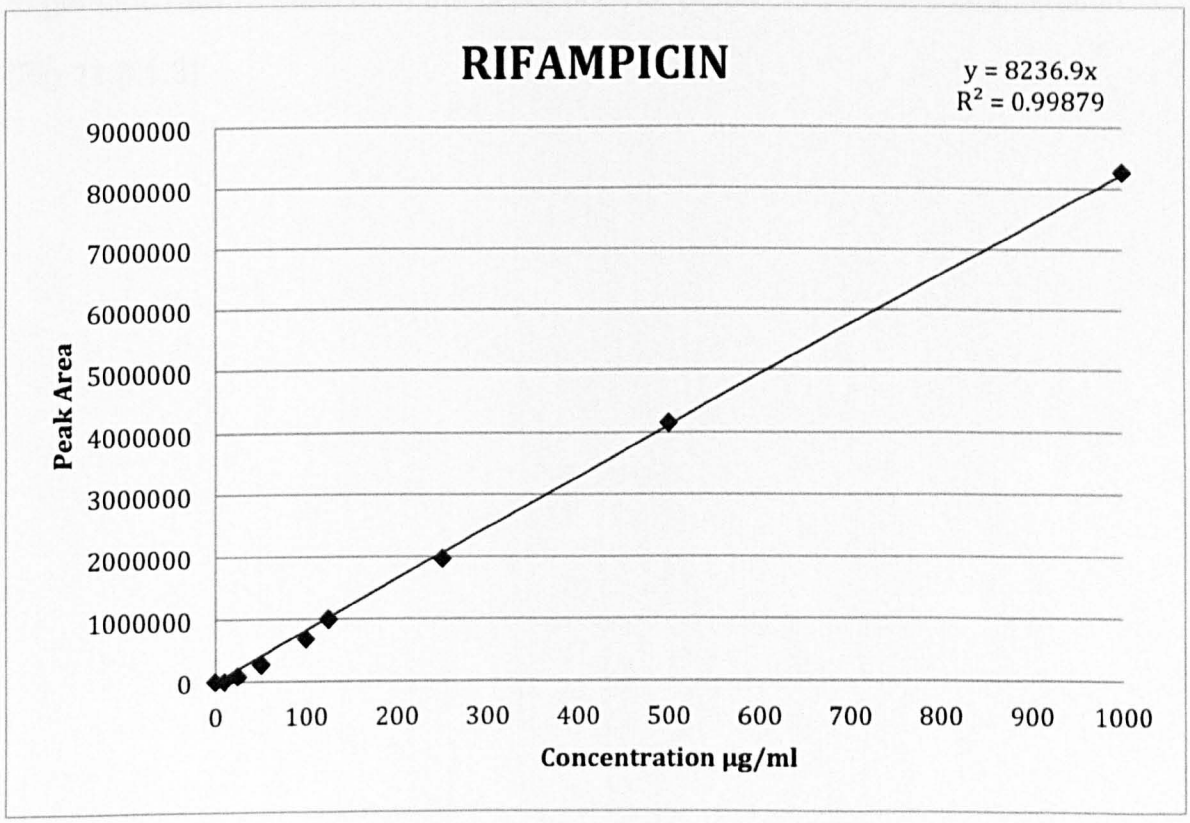


Fig 11.3.1.2: Graphs illustrating the relationship between antibiotic concentration and peak area.

When examining drug release the pellets remained in the Eppendorf tube after PBS was aspirated and replaced; therefore, a possible concern was that antibiotics may remain in the Eppendorf or be taken up by the Eppendorf tube material despite PBS change, so that any antibiotic detected would not be there as a result of new release from the pellet but may be there simply as a "left over" attached to the tube. Therefore, rifampicin and clindamycin solutions (50 µg/mL) were placed into an Eppendorf and left overnight. The solution was then aspirated, and fresh PBS placed into the Eppendorf. This PBS was then analysed as rinse 1, and repeated as rinse 2 and 3.

Even on rinse 1, there was no clear peak for either clindamycin or rifampicin, suggesting that any drug seen in the PBS exposed to antibiotic pellet was due to genuine new release from the pellet and not result of residual contamination (Fig 11.3.1.3).

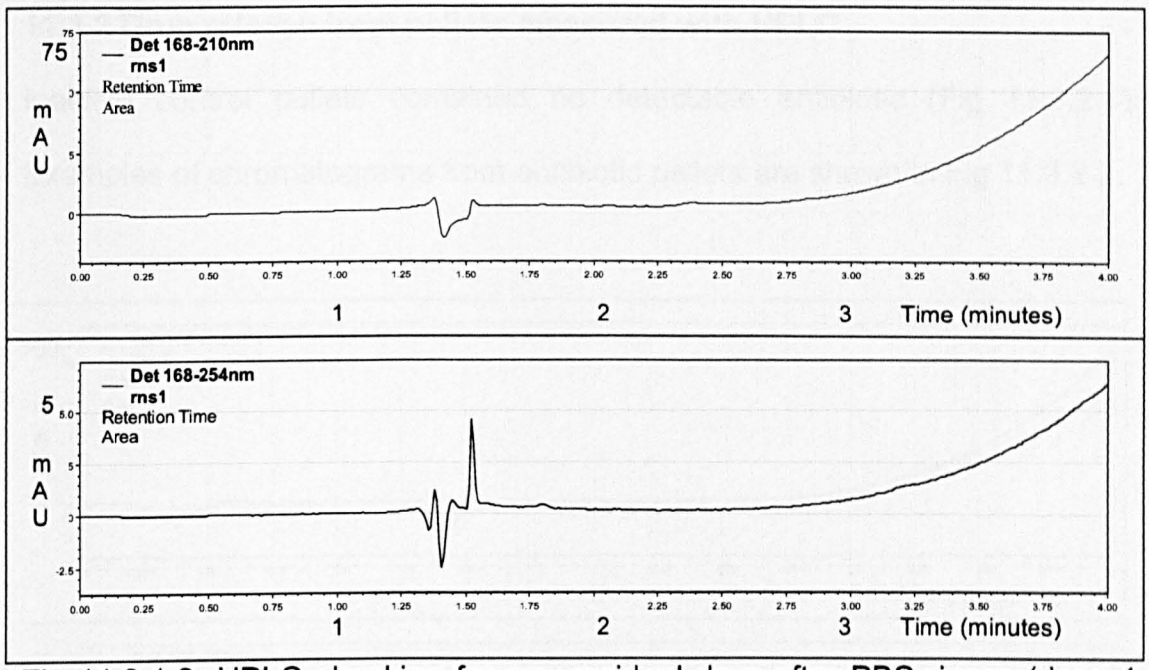


Fig 11.3.1.3: HPLC checking for any residual drug after PBS rinses (rinse 1 shown). Top row shows absorption at 210 nm (clindamycin) and bottom absorption at 254 nm (rifampicin).

11.3.2 Drug release from pellets measured with HPLC

Inactive control pellets contained no detectable antibiotic (Fig 11.3.2.1). Examples of chromatograms from antibiotic pellets are shown in Fig 11.3.2.2.

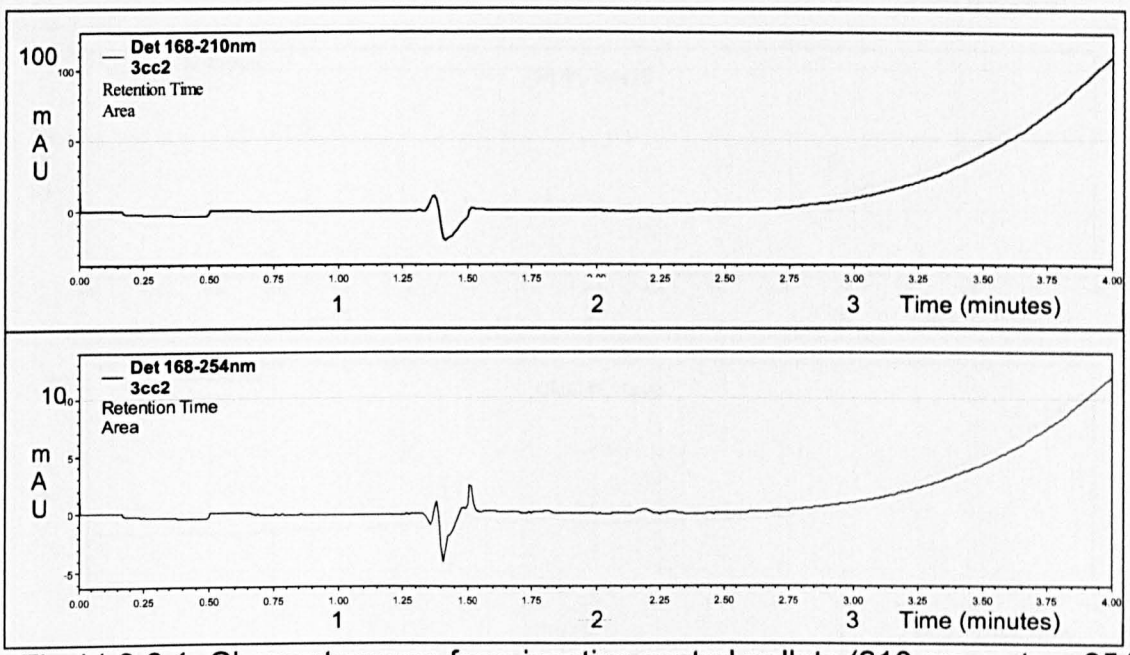
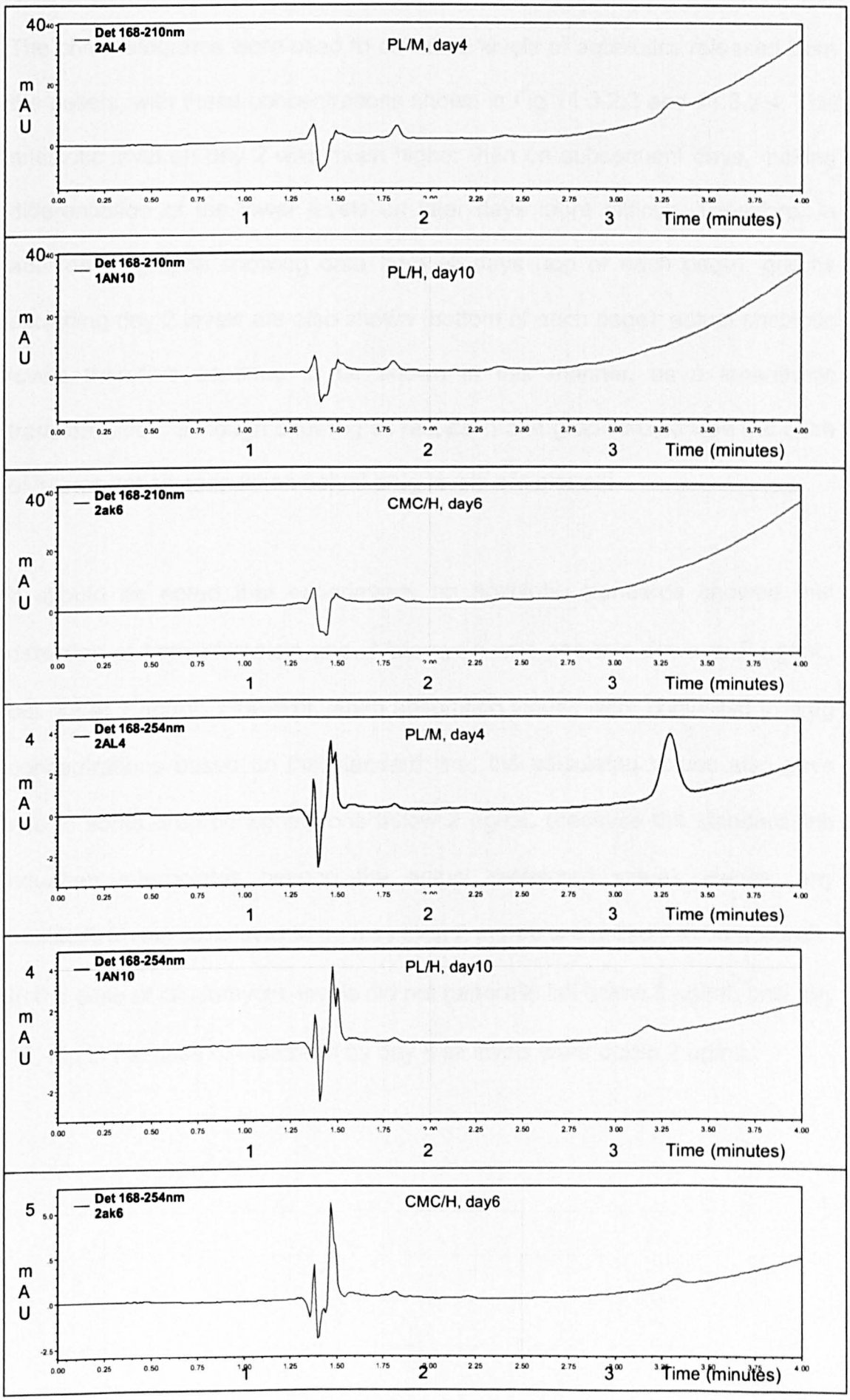


Fig 11.3.2.1: Chromatograms from inactive control pellets (210 nm on top, 254 nm on bottom)

Fig 11.3.2.2 (over page): Example chromatograms from antibiotic pellets, 210 nm for clindamycin (top 3 graphs), 254 nm for rifampicin (bottom 3).


Det 168-210nm
2AL4
PL/M, day4
mAU
Time (minutes)
Det 168-210nm
1AN10
PL/H, day10
mAU
Time (minutes)
Det 168-210nm
2ak6
CMC/H, day6
mAU
Time (minutes)
Det 168-254nm
2AL4
PL/M, day4
mAU
Time (minutes)
Det 168-254nm
1AN10
PL/H, day10
mAU
Time (minutes)
Det 168-254nm
2ak6
CMC/H, day6
mAU
Time (minutes)

The chromatograms were used to calculate levels of antibiotics released from the pellets, with these concentrations shown in Fig 11.3.2.3 and 11.3.2.4. The antibiotic level on day 2 was much higher than on subsequent days, making differentiation of the lower levels on later days more difficult. Therefore, in addition to graphs showing data from all days (top of each page), graphs excluding day 2 levels are also shown (bottom of each page); actual antibiotic levels therefore continue to be shown in this manner, as a logarithmic transformation, although showing all results in one graph would lose the ease of interpretation seen when actual drug levels are plotted.

It should be noted that experiments on antibiotic standards showed that detection of both rifampicin and clindamycin was possible down to 2 μg/mL, but not at 1 μg/mL. However, when absorption values were converted to drug concentrations based on the standard line, the calculated values also gave rise to some drug concentrations below 2 μg/mL (because the standard line equation interpolates beyond the actual measured value). Hence, any antibiotic levels calculated to be less than 2 μg/mL are unlikely to be accurate. In the case of clindamycin, levels did not generally fall below 2 μg/mL until day 16, but in the case of rifampicin by day 6 all levels were below 2 μg/mL.

The following series of graphs shows levels of clindamycin (Fig 11.3.2.3) and rifampicin (Fig 11.3.2.4) released from the pellets. All the different pellets are shown together on the same graph first (a), followed by effect of variation of drug level for CMC (b) and pluronic (c), then comparison of carrier choice whilst keeping drug level constant (d, e, f), and finally looking at effect of processing variation on a CMC pellet and changing CMC viscosity from high to medium viscosity (g). Composition of different pellets is shown in Table 11.1.1. Error bars represent standard deviation. To give an indication of how these levels relate to the Minimum Inhibitory Concentration (MIC) levels of the *S aureus* used in biofilm experiments, each graph also contains a line indicating level equivalent to 1000xMIC. Statistical significance is shown by the presence of a star on the graphs b-f, although due to small levels in the case of rifampicin no statistical analysis was performed beyond day 4; p values for the various comparisons are shown in Table 11.3.2.1 and 11.3.2.2.

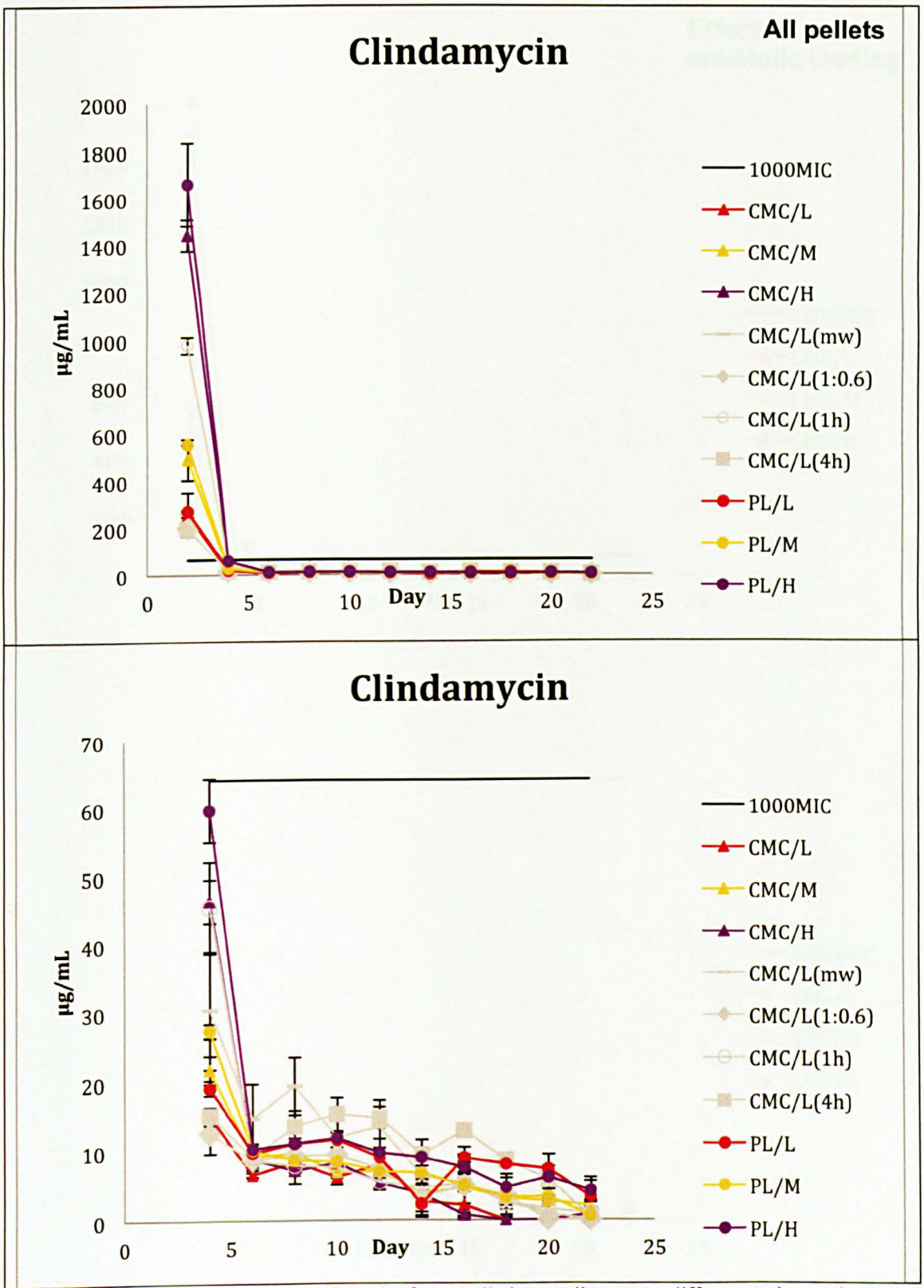


Fig 11.3.2.3a: Clindamycin levels from all the pellets on different days. Lower graph excludes day 2 data. Pellet composition is shown in Table 11.1.1. Error bars indicate Standard Deviation

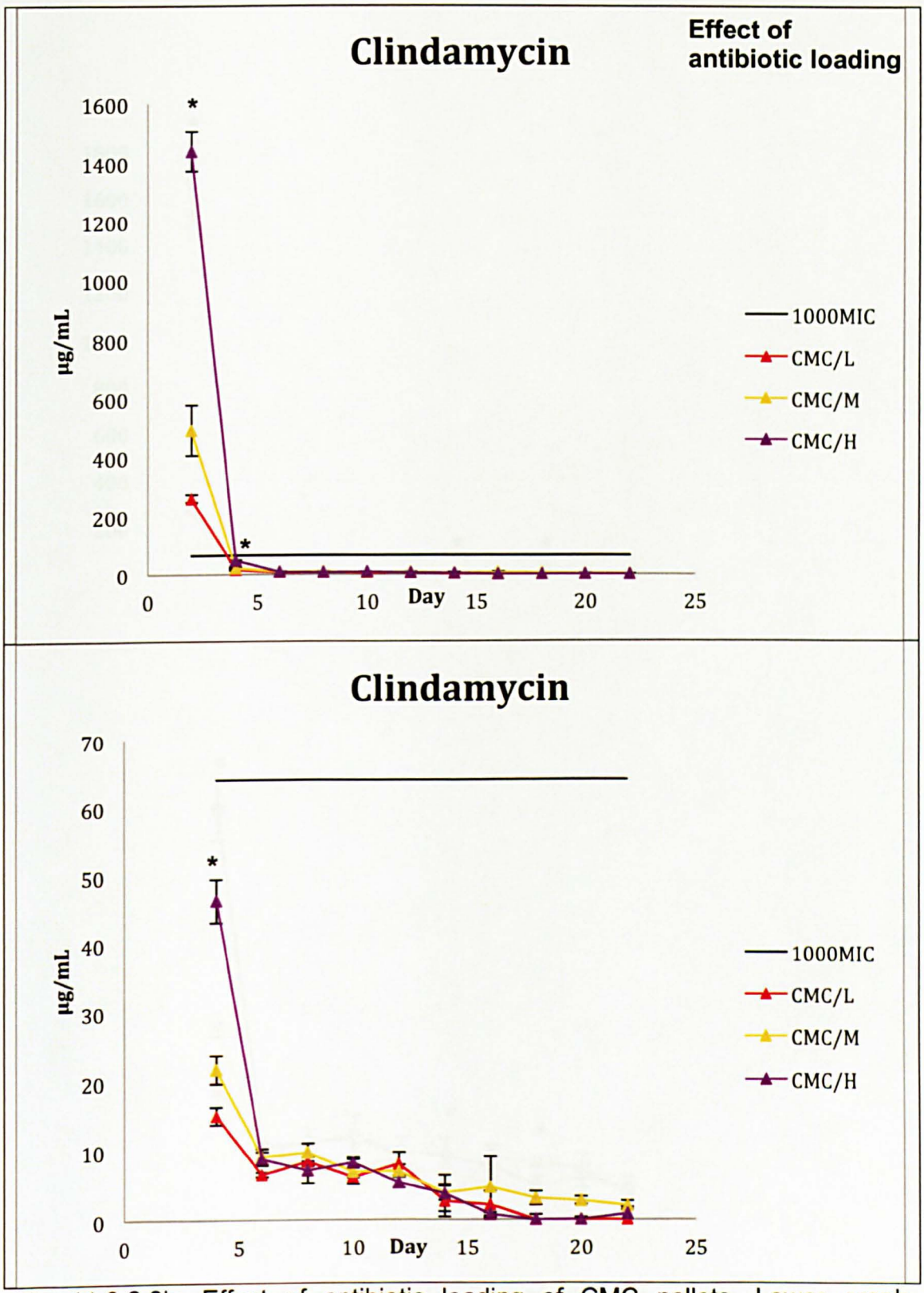


Fig 11.3.2.3b: Effect of antibiotic loading of CMC pellets. Lower graph excludes day 2 data. Pellet composition is shown in Table 11.1.1. Error bars indicate Standard Deviation, and stars the day on which the levels were significantly different.

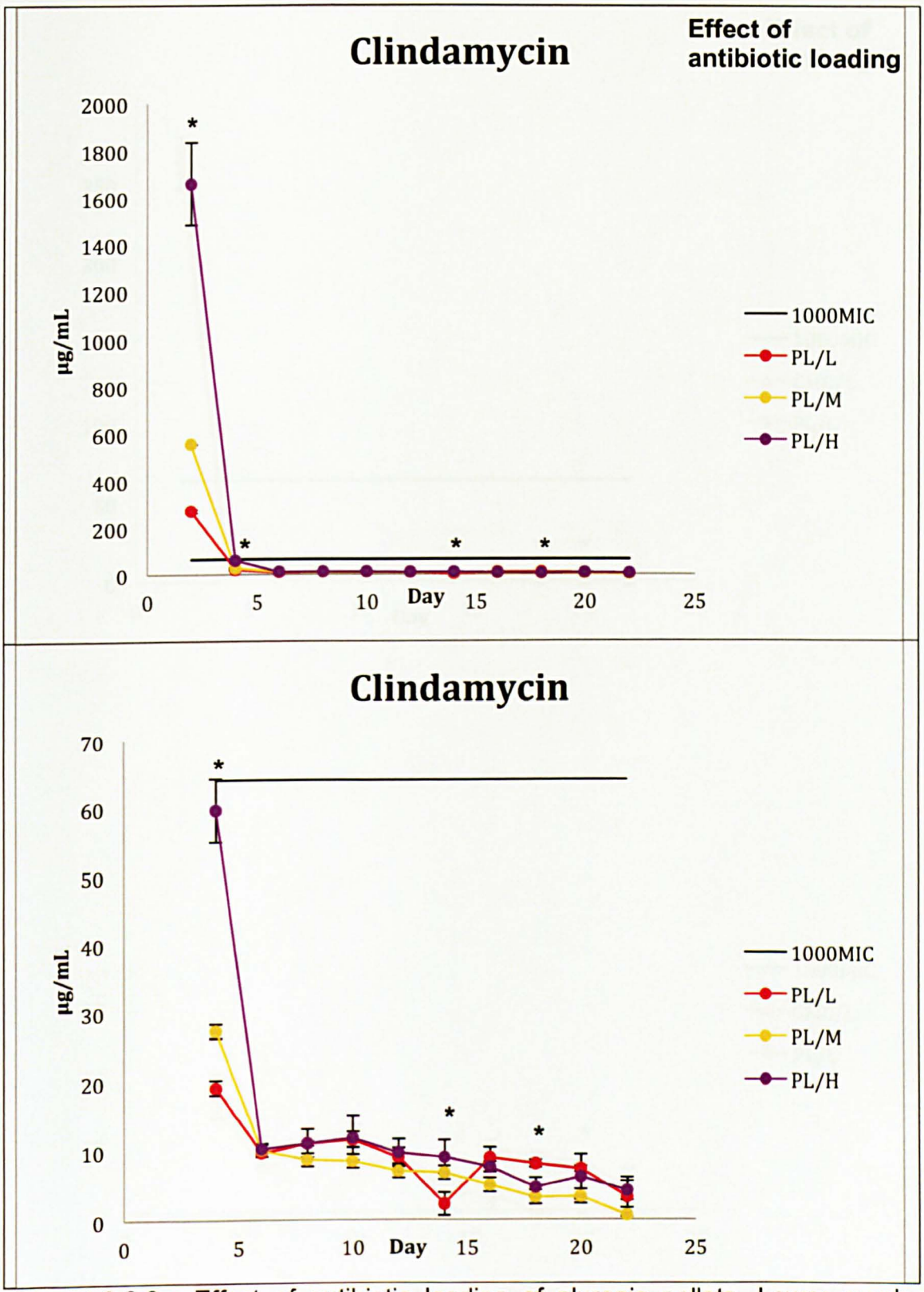


Fig 11.3.2.3c: Effect of antibiotic loading of pluronic pellets. Lower graph excludes day 2 data. Pellet composition is shown in Table 11.1.1. Error bars indicate Standard Deviation, and stars the day on which the levels were significantly different.

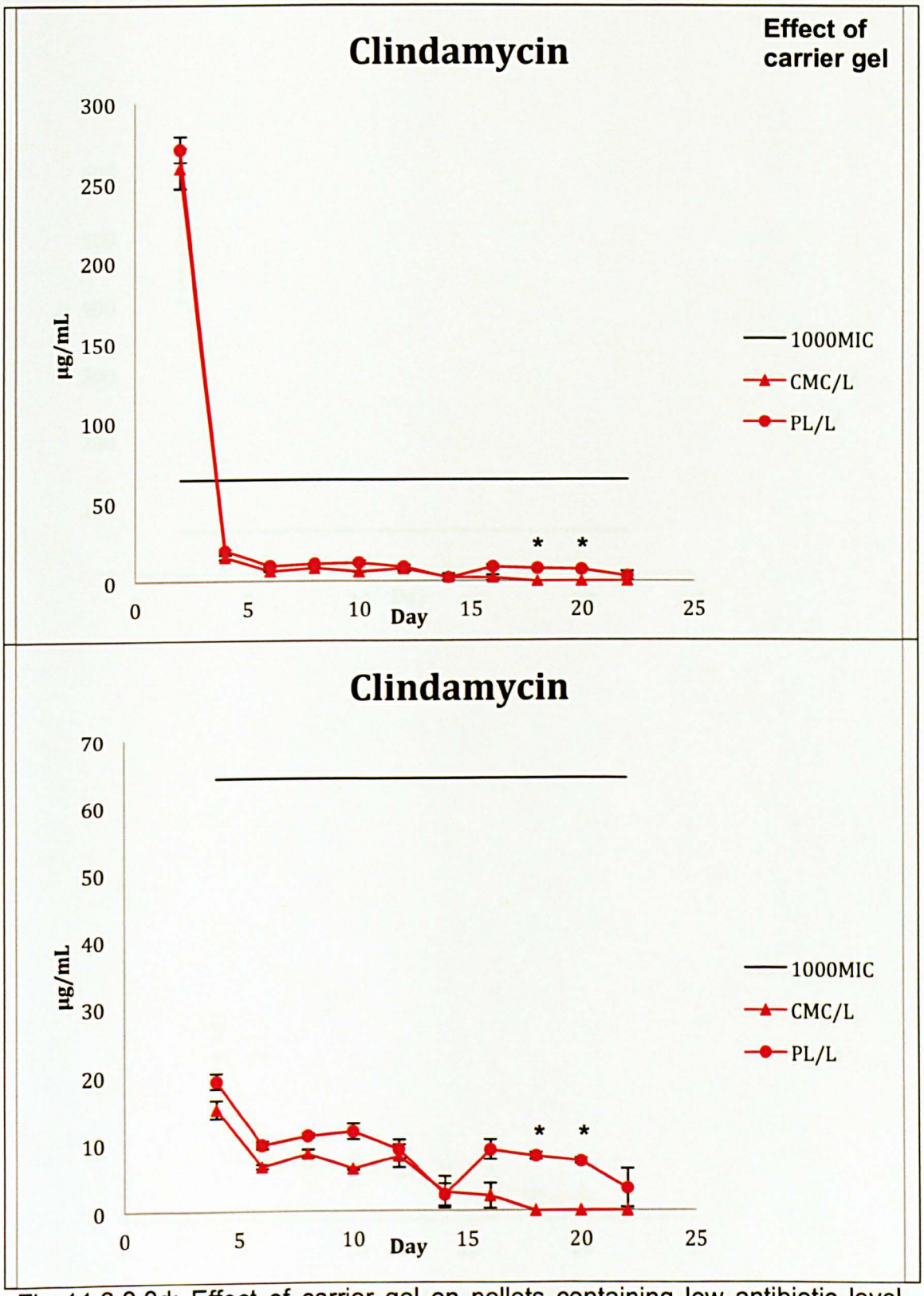


Fig 11.3.2.3d: Effect of carrier gel on pellets containing low antibiotic level. Lower graph excludes day 2 data. Error bars indicate Standard Deviation, and stars the day on which the levels were significantly different.

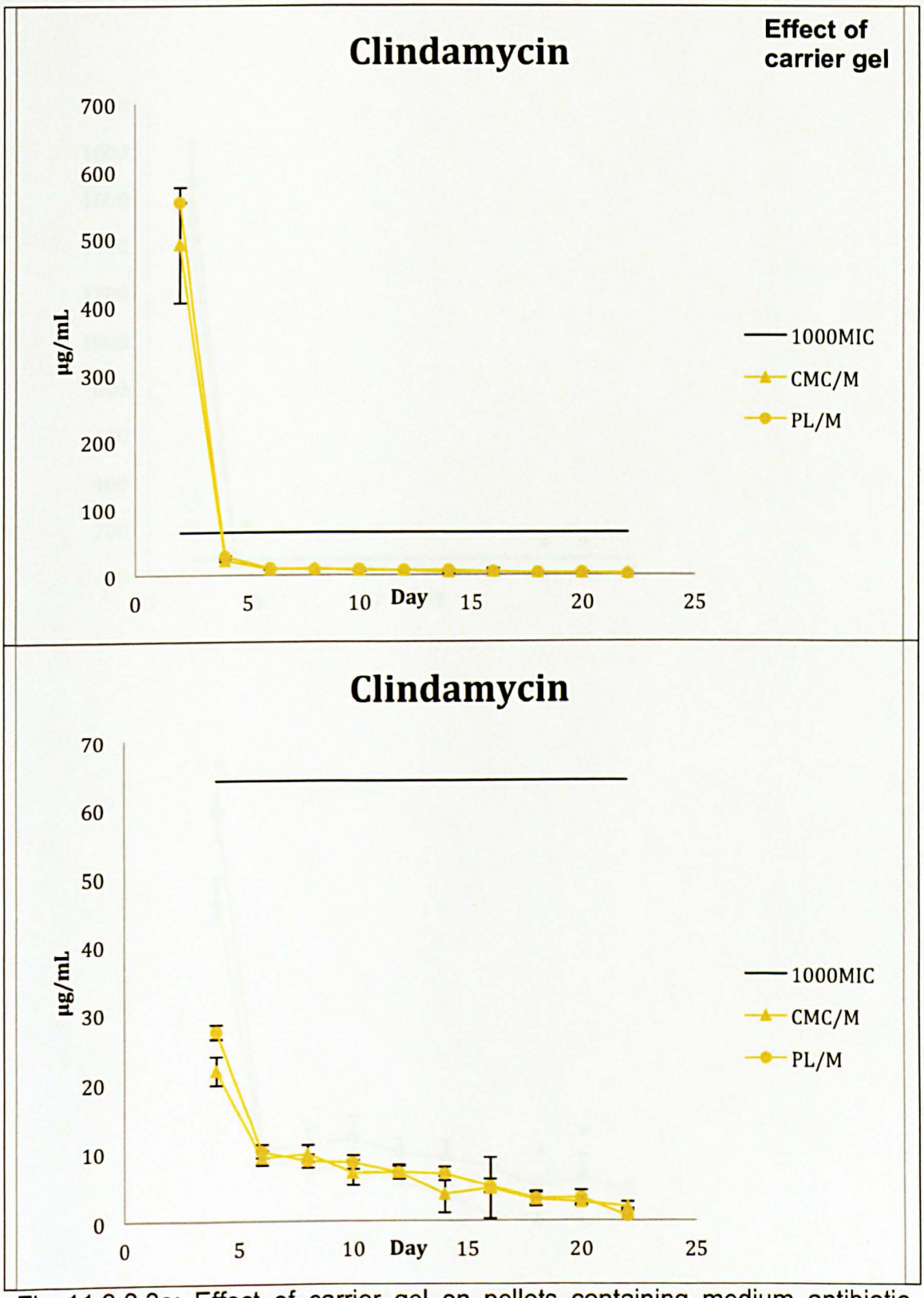


Fig 11.3.2.3e: Effect of carrier gel on pellets containing medium antibiotic level. Lower graph excludes day 2 data. Error bars indicate Standard Deviation. Values were not significantly different on any day.

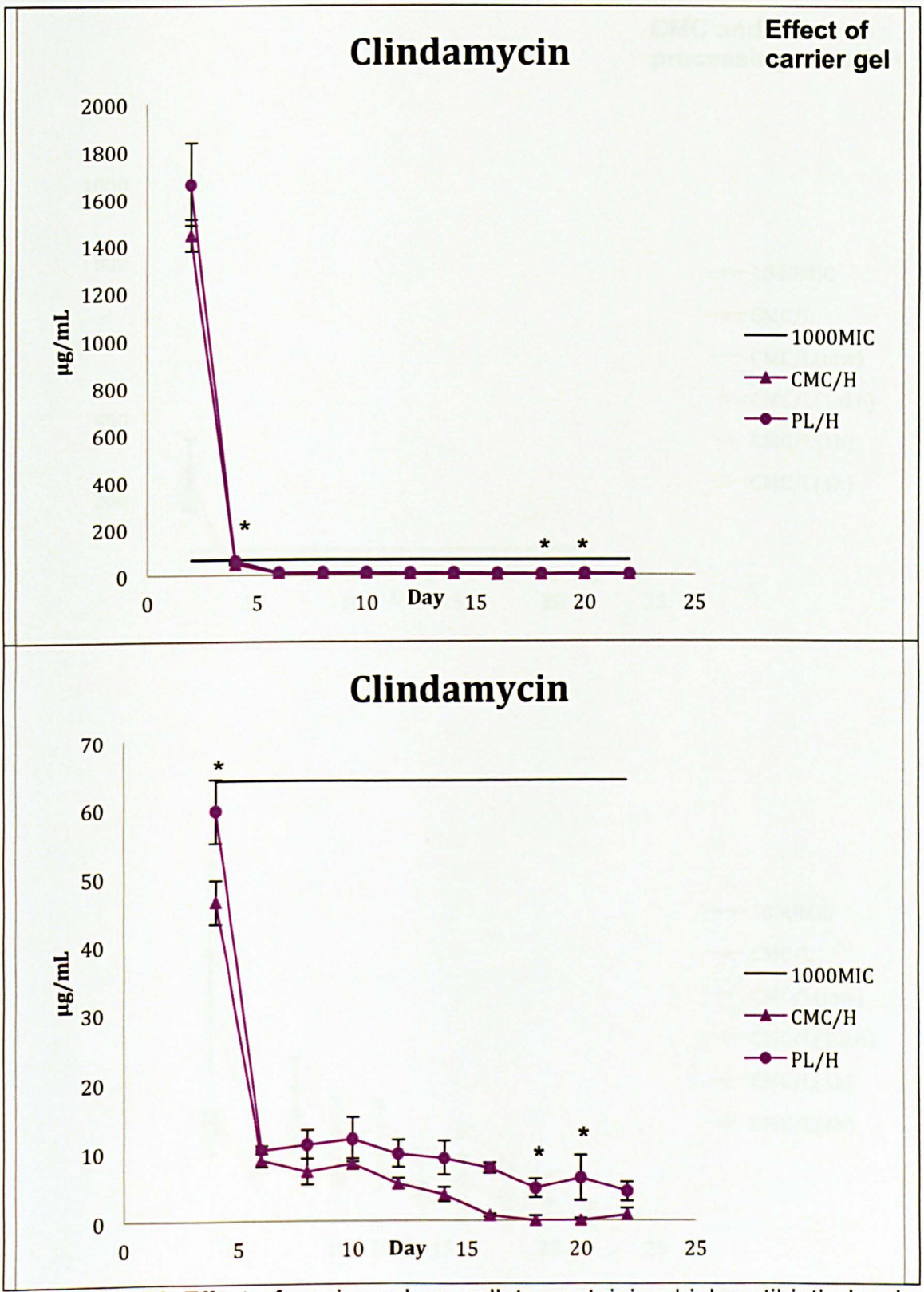


Fig 11.3.2.3f: Effect of carrier gel on pellets containing high antibiotic level. Lower graph excludes day 2 data. Error bars indicate Standard Deviation, and stars the day on which the levels were significantly different.

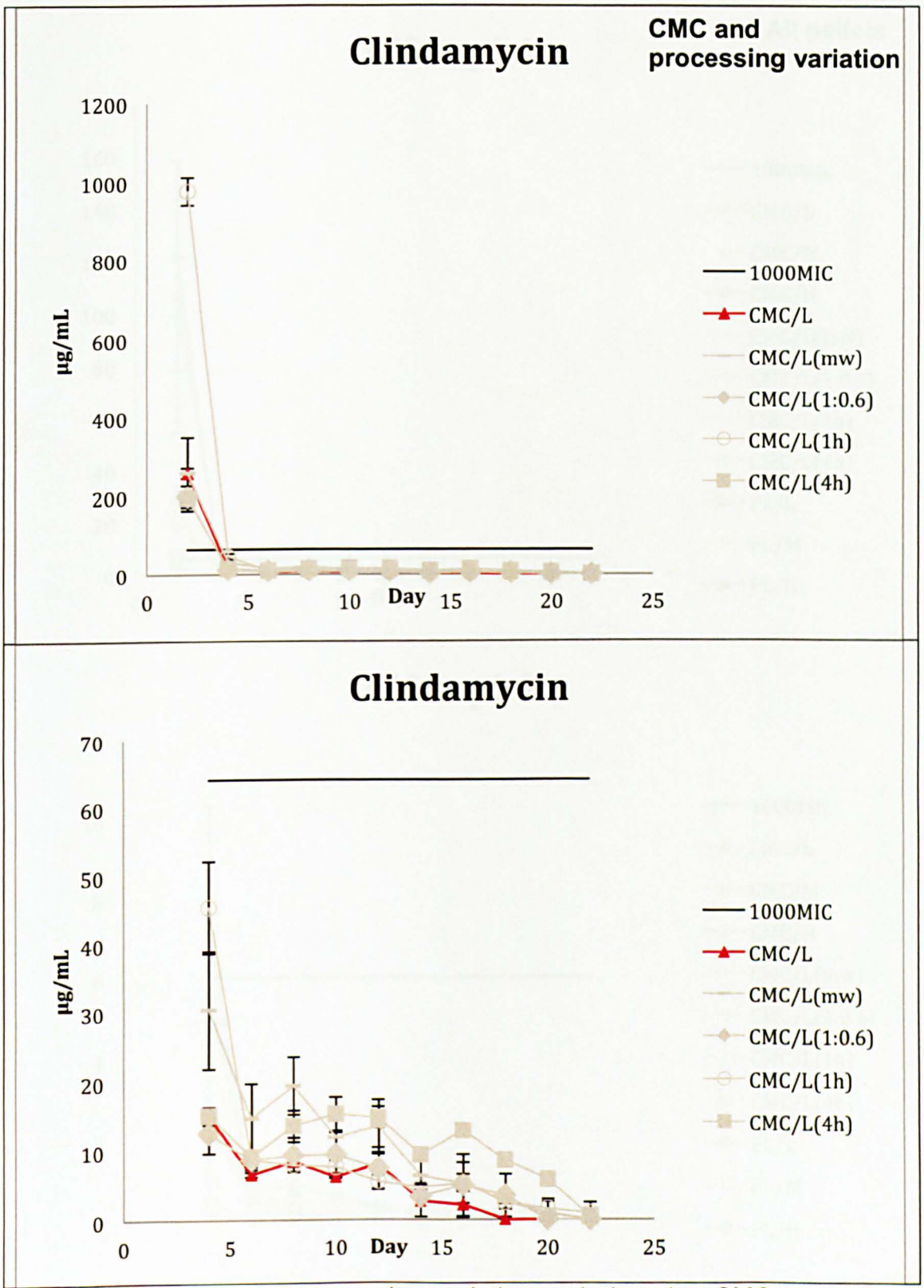


Fig 11.3.2.3g: Effect of processing variation and changing CMC to medium viscosity instead of high viscosity. Lower graph excludes day 2 data. Error bars indicate Standard Deviation.

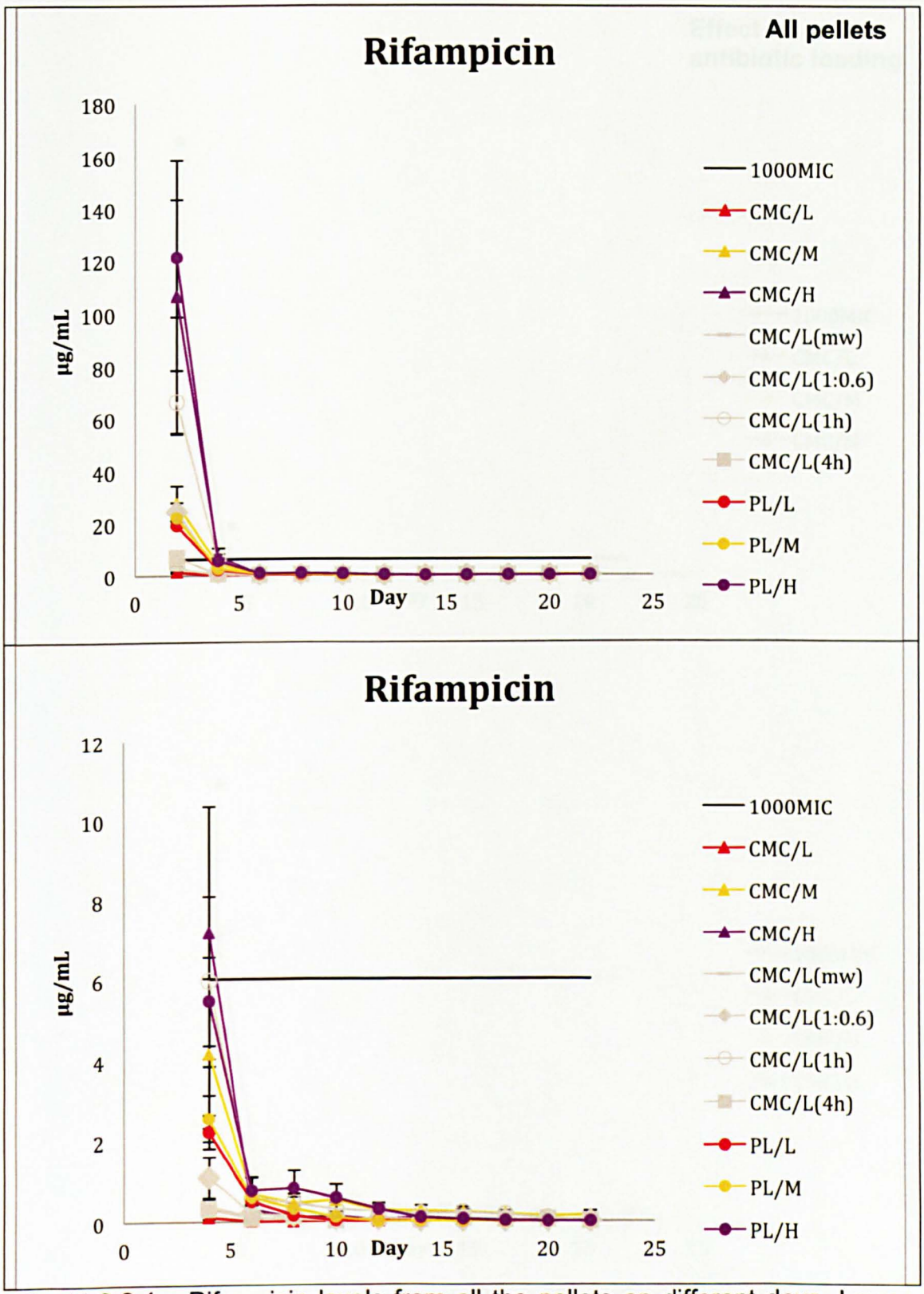


Fig 11.3.2.4a: Rifampicin levels from all the pellets on different days. Lower graph excludes day 2 data. Pellet composition is shown in Table 11.1.1. Error bars indicate Standard Deviation.

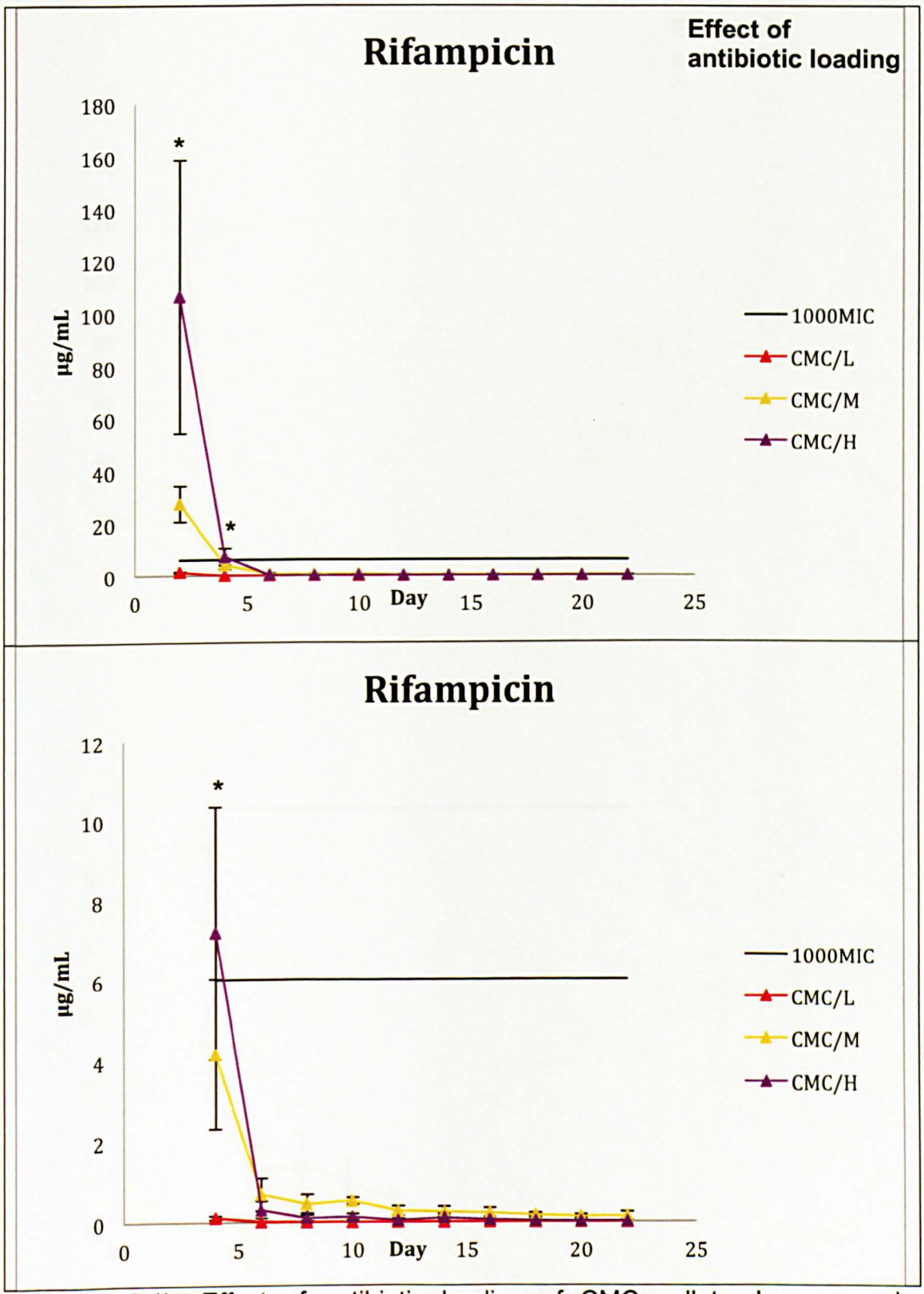


Fig 11.3.2.4b: Effect of antibiotic loading of CMC pellets. Lower graph excludes day 2 data. Pellet composition is shown in Table 11.1.1. Error bars indicate Standard Deviation, and stars the day on which the levels were significantly different.

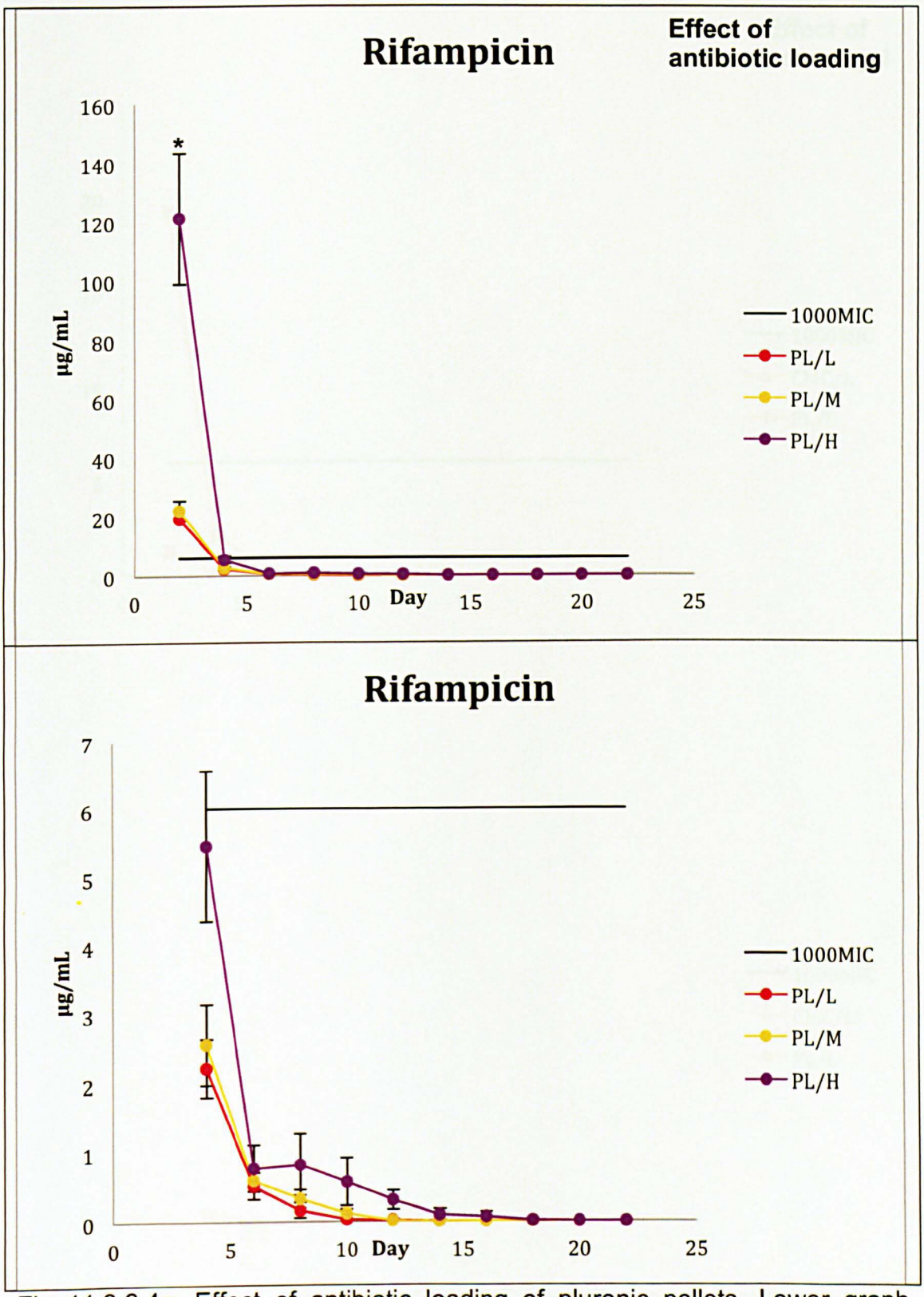


Fig 11.3.2.4c: Effect of antibiotic loading of pluronic pellets. Lower graph excludes day 2 data. Pellet composition is shown in Table 11.1.1. Error bars indicate Standard Deviation, and stars the day on which the levels were significantly different.

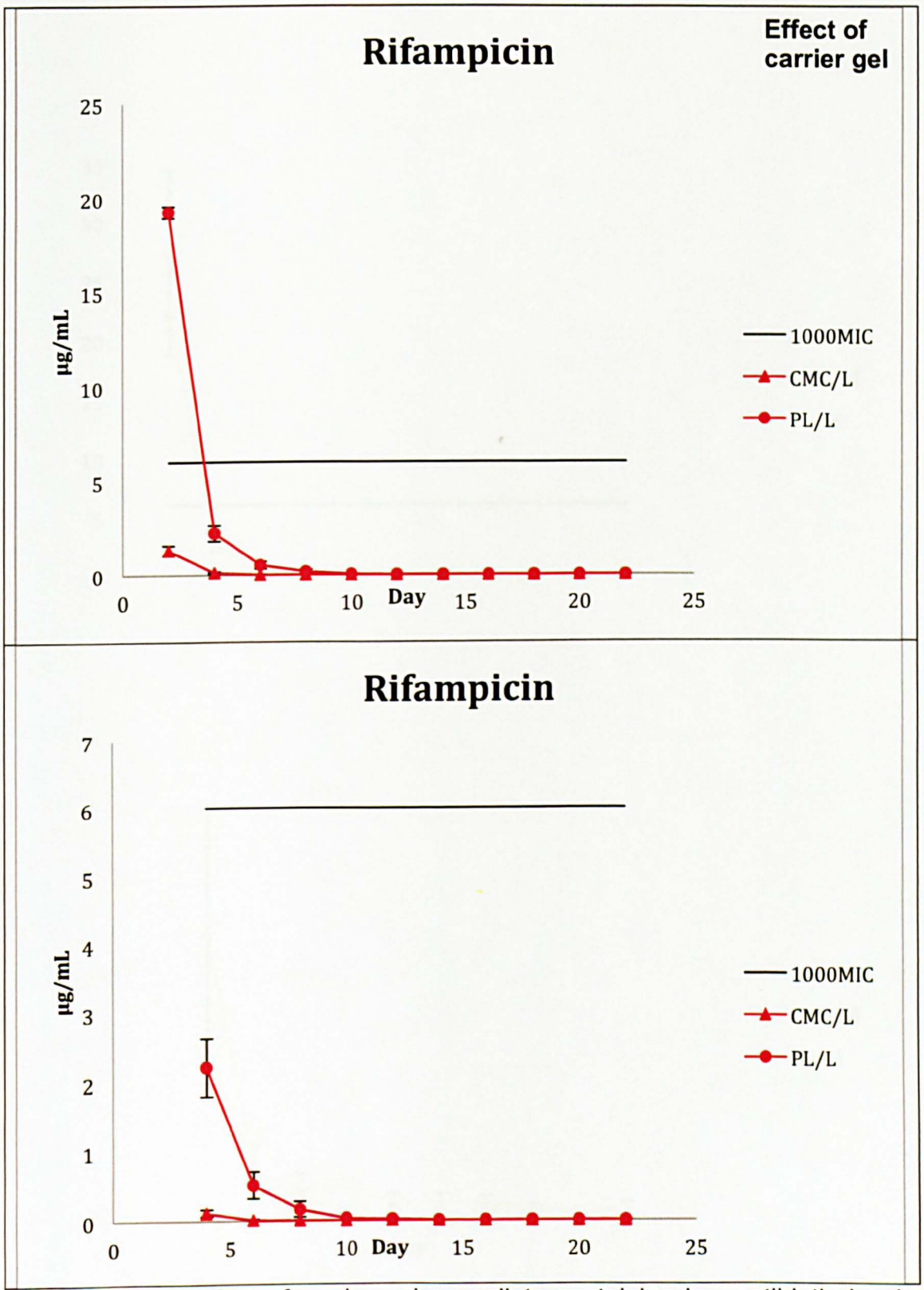


Fig 11.3.2.4d: Effect of carrier gel on pellets containing low antibiotic level. Lower graph excludes day 2 data. Error bars indicate Standard Deviation. The levels were not significantly different on any day.

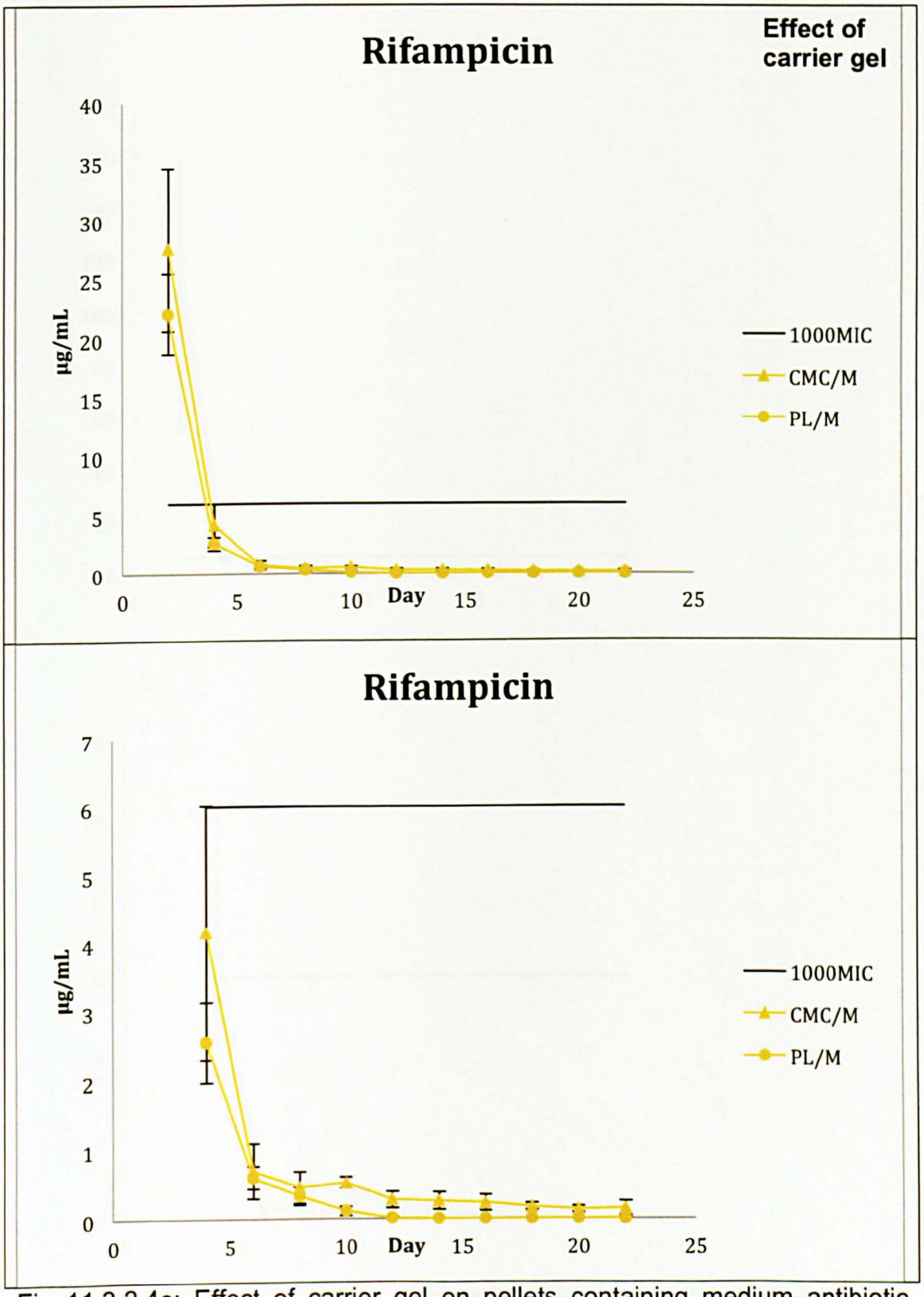


Fig 11.3.2.4e: Effect of carrier gel on pellets containing medium antibiotic level. Lower graph excludes day 2 data. Error bars indicate Standard Deviation. The levels were not significantly different on any day.

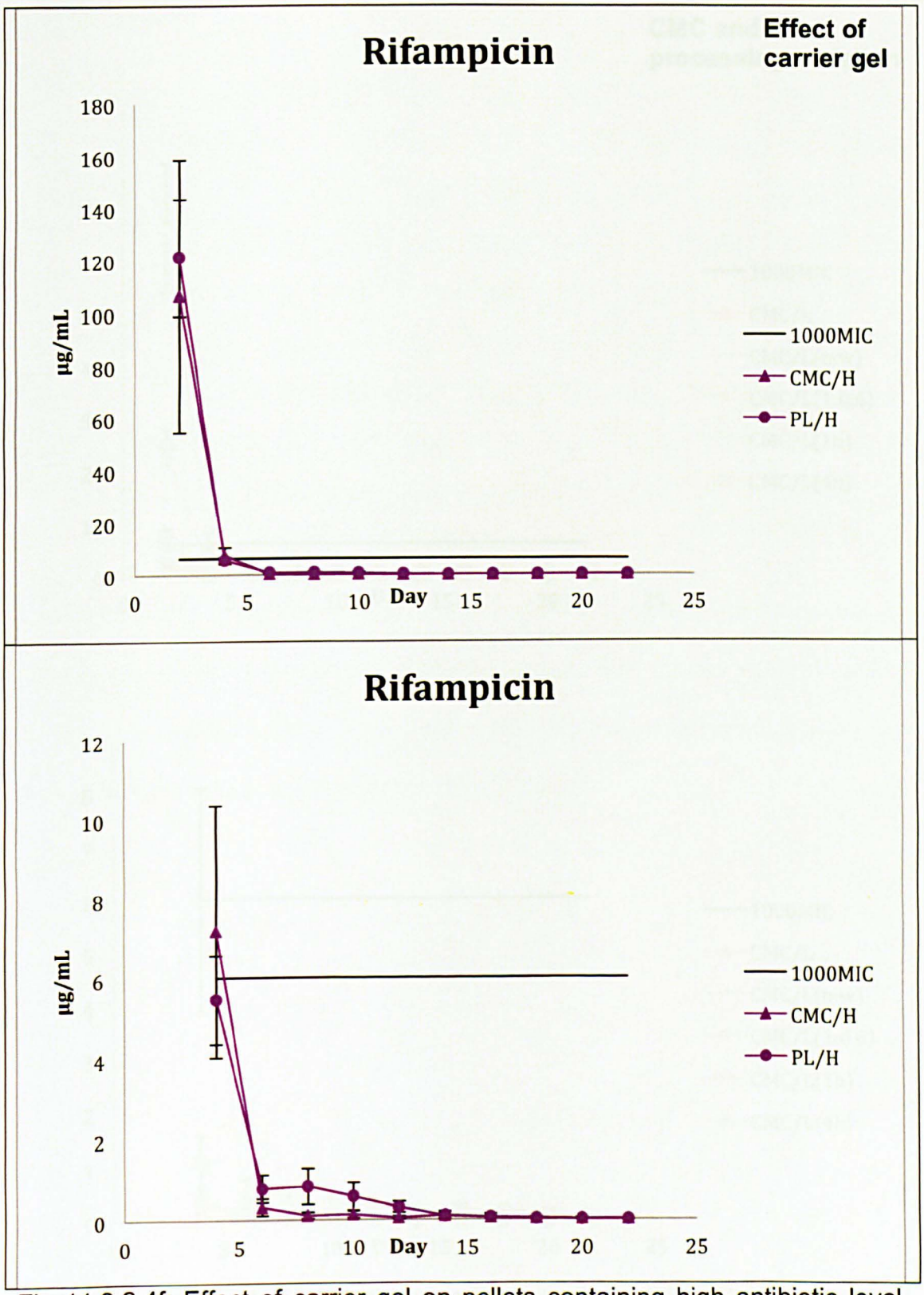


Fig 11.3.2.4f: Effect of carrier gel on pellets containing high antibiotic level. Lower graph excludes day 2 data. Error bars indicate Standard Deviation. The levels were not significantly different on any day.

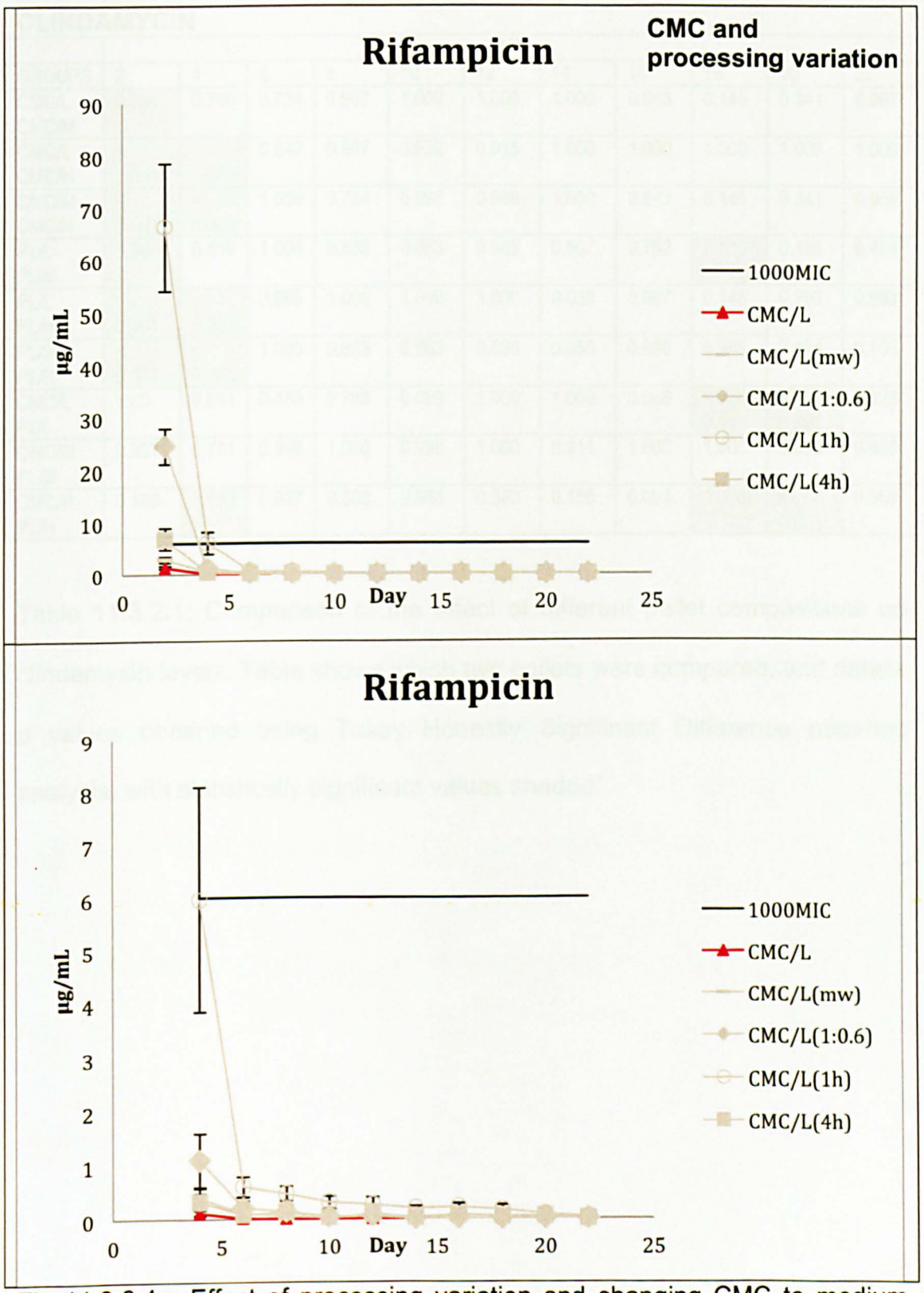


Fig 11.3.2.4g: Effect of processing variation and changing CMC to medium viscosity instead of high viscosity. Lower graph excludes day 2 data. Error bars indicate Standard Deviation.

CLINDAMYCIN

	DAY										
GROUPS	2	4	6	8	10	12	14	16	18	20	22
CMC/L CMC/M	0.034	0.740	0.724	0.997	1.000	1.000	1.000	0.943	0.145	0.341	0.697
CMC/L CMC/H	< 0.001	< 0.001	0.842	0.997	0.932	0.915	1.000	1.000	1.000	1.000	1.000
CMC/M CMC/H	< 0.001	< 0.001	1.000	0.784	0.998	0.998	1.000	0.643	0.145	0.341	0.956
PL/L PL/M	0.006	0.459	1.000	0.883	0.663	0.965	0.567	0.762	0.036	0.105	0.404
PL/L PL/H	< 0.001	< 0.001	0.999	1.000	1.000	1.000	0.038	0.997	0.145	0.993	0.993
PL/M PL/H	< 0.001	< 0.001	1.000	0.883	0.663	0.836	0.955	0.988	0.985	0.404	0.105
CMC/L PL/L	1.00	0.961	0.453	0.784	0.089	1.000	1.000	0.068	< 0.001	< 0.001	0.123
CMC/M PL/M	0.987	0.791	0.999	1.000	0.998	1.000	0.911	1.000	1.000	0.999	0.955
CMC/H PL/H	0.106	0.033	0.927	0.303	0.663	0.380	0.155	0.093	0.005	< 0.001	0.069

Table 11.3.2.1: Comparison of the effect of different pellet compositions on clindamycin levels. Table shows which two pellets were compared, and details p values obtained using Tukey Honestly Significant Difference post-hoc analysis, with statistically significant values shaded.

RIFAMPICIN

	DAY	
GROUPS	2	4
CMC/L CMC/M	0.767	0.046
CMC/L CMC/H	< 0.001	< 0.001
CMC/M CMC/H	0.001	0.336
PL/L PL/M	1.000	1.000
PL/L PL/H	< 0.001	0.336
PL/M PL/H	< 0.001	0.336
CMC/L PL/L	0.967	0.653
CMC/M PL/M	1.000	0.808
CMC/H PL/H	0.990	0.808

Table 11.3.2.2: Comparison of the effect of different pellet compositions on rifampicin levels on days 2 and 4 (later values were not subject to statistical analysis due to small antibiotic levels). Table shows which two pellets were compared, and details p values obtained using Tukey Honestly Significant Difference post-hoc analysis, with statistically significant values shaded.

Cumulative per cents of drugs release are shown in Fig 11.3.2.5.

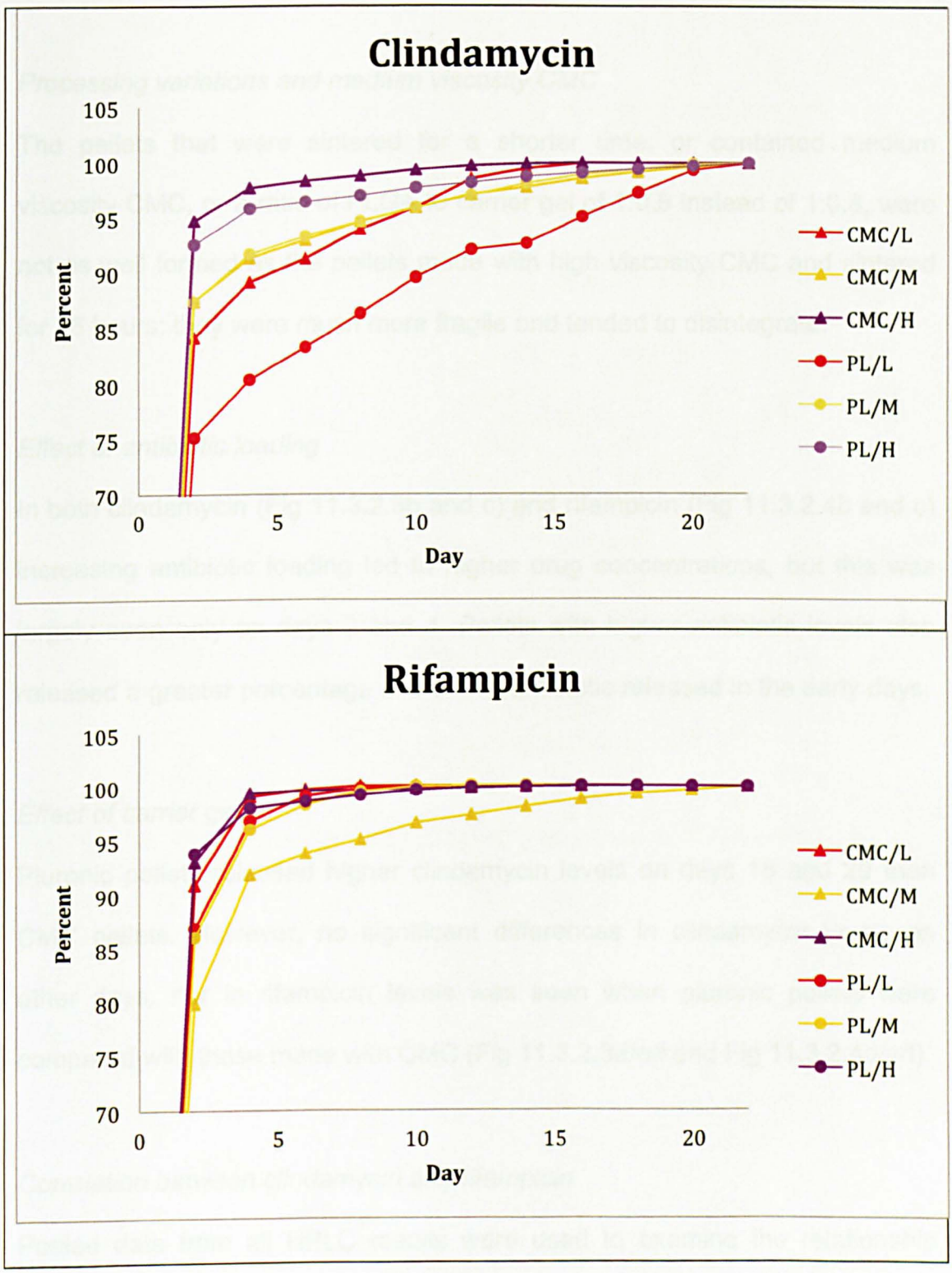


Fig 11.3.2.5: Cumulative percentage of clindamycin (top) and rifampicin (bottom) released from antibiotic pellets over 22 days.

Processing variations and medium viscosity CMC

The pellets that were sintered for a shorter time, or contained medium viscosity CMC, or a ratio of PLGA to carrier gel of 1:0.6 instead of 1:0.8, were not as well formed as the pellets made with high viscosity CMC and sintered for 16 hours; they were much more fragile and tended to disintegrate.

Effect of antibiotic loading

In both clindamycin (Fig 11.3.2.3b and c) and rifampicin (Fig 11.3.2.4b and c) increasing antibiotic loading led to higher drug concentrations, but this was largely seen only on days 2 and 4. Pellets with higher antibiotic levels also released a greater percentage of the total antibiotic released in the early days.

Effect of carrier gel

Pluronic pellets released higher clindamycin levels on days 18 and 20 than CMC pellets. However, no significant differences in clindamycin levels on other days, nor in rifampicin levels was seen when pluronic pellets were compared with those made with CMC (Fig 11.3.2.3d/e/f and Fig 11.3.2.4d/e/f).

Correlation between clindamycin and rifampicin

Pooled data from all HPLC results were used to examine the relationship between rifampicin and clindamycin levels from the same pellet (Fig 11.3.2.5). The Pearson correlation coefficient was 0.955 ($p<0.001$), indicating a strong correlation.

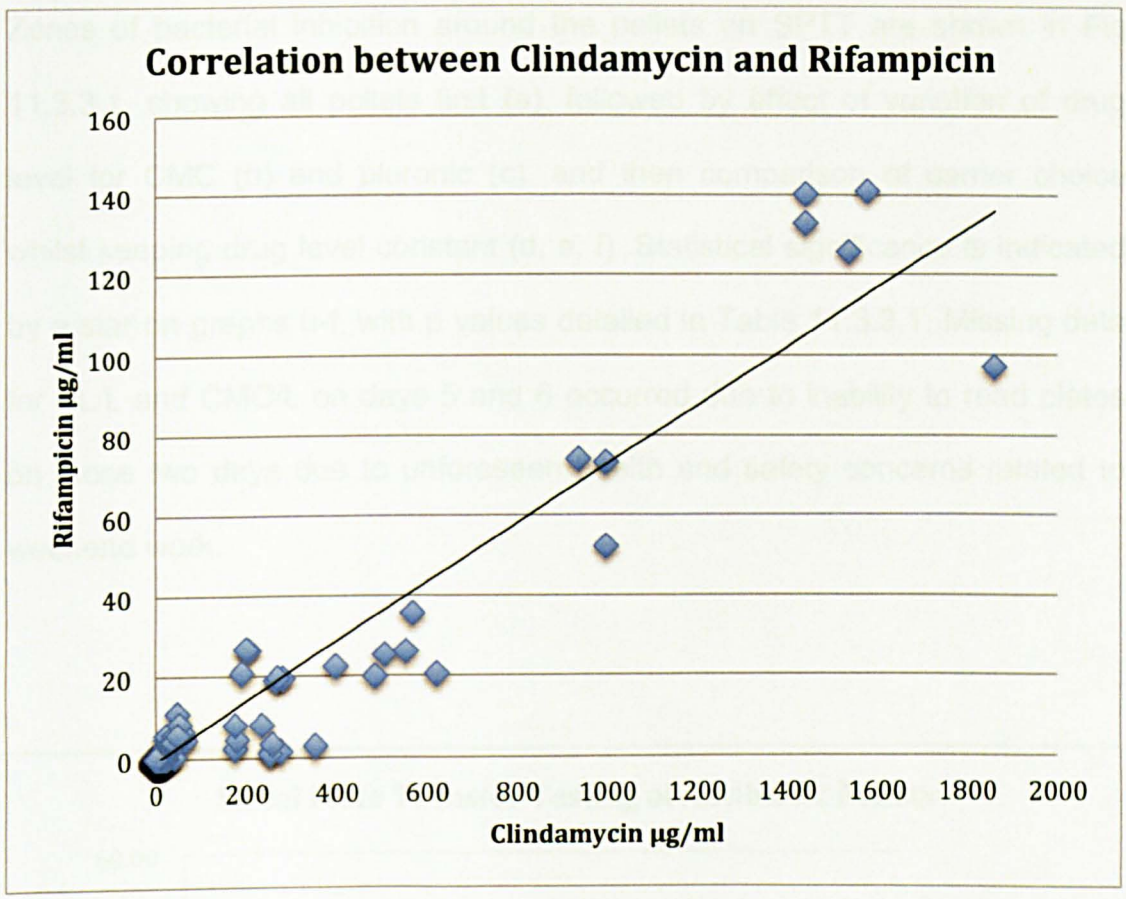


Fig 11.3.2.5: Correlation between rifampicin and clindamycin levels.

11.3.3 Antibiotic release measured using serial plate transfer test

Zones of bacterial inhibition around the pellets on SPTT are shown in Fig 11.3.3.1, showing all pellets first (a), followed by effect of variation of drug level for CMC (b) and pluronic (c), and then comparison of carrier choice whilst keeping drug level constant (d, e, f). Statistical significance is indicated by a star on graphs b-f, with p values detailed in Table 11.3.3.1. Missing data for PL/L and CMC/L on days 5 and 6 occurred due to inability to read plates on those two days due to unforeseen health and safety concerns related to weekend work.

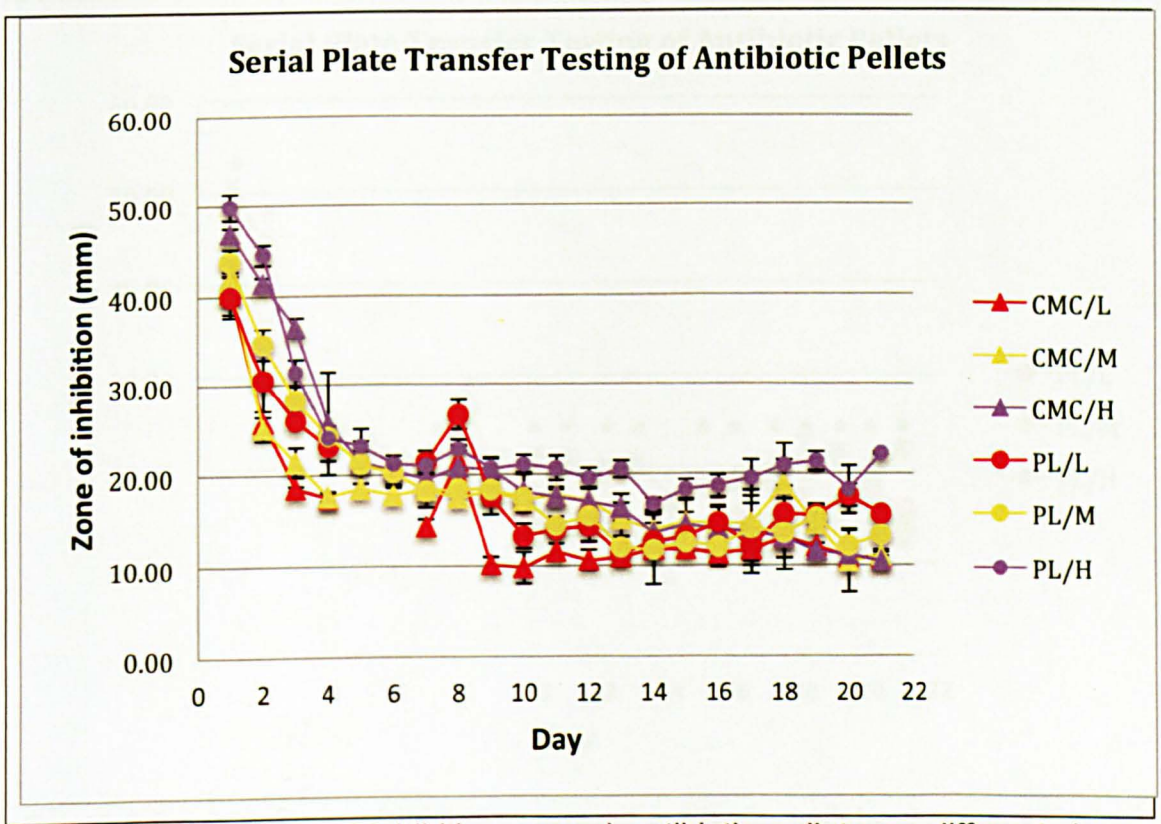


Fig 11.3.3.1a: Zone of inhibition around antibiotic pellets on different days. Pellet composition is shown in Table 11.1.1. Error bars indicate Standard Deviation.

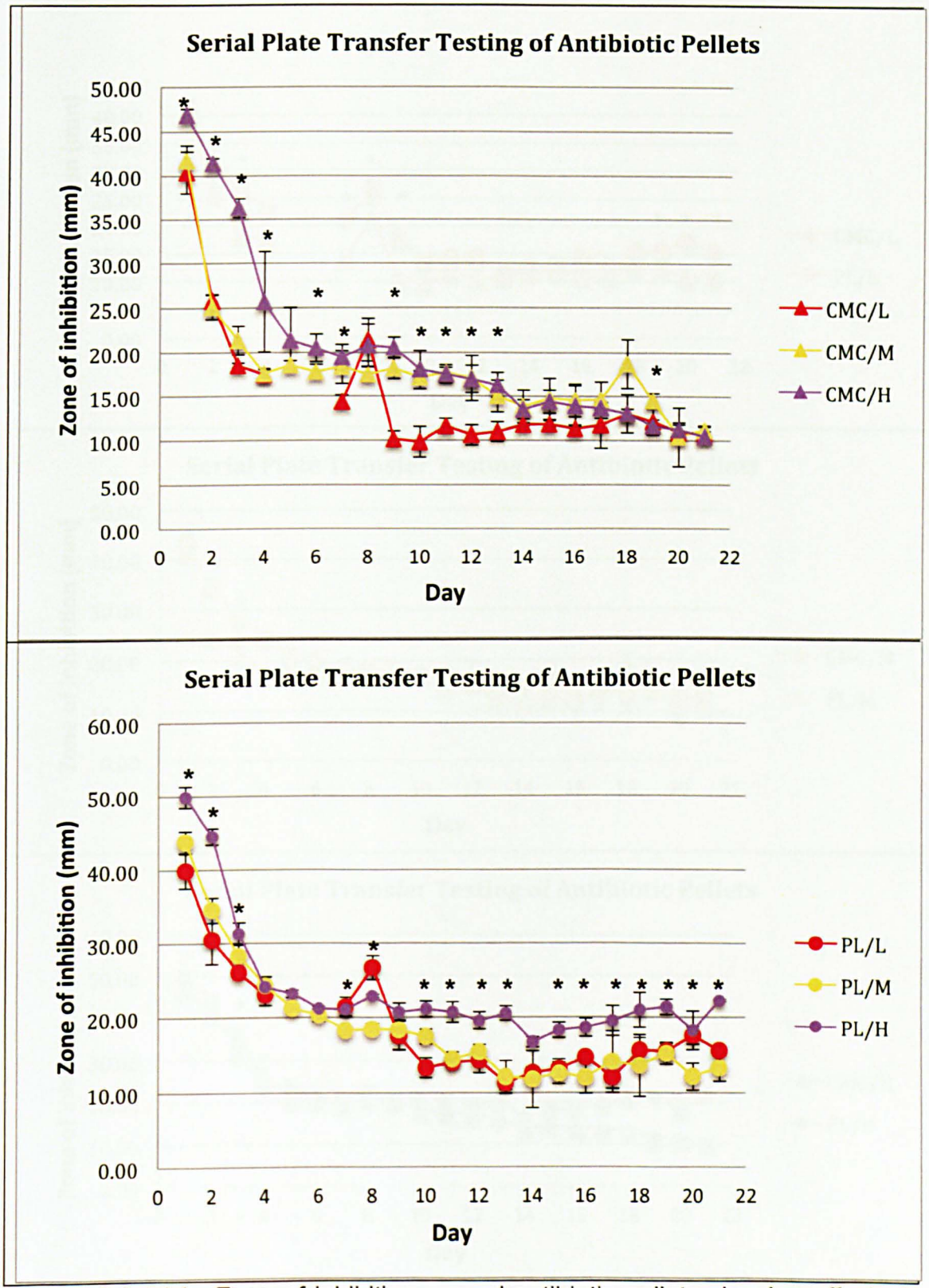


Fig 11.3.3.1 b/c: Zone of inhibition around antibiotic pellets showing effect of drug loading in CMC pellets (b, top) and pluronic pellets (c, bottom). Error bars indicate Standard Deviation, and stars the day on which the different levels were statistically significant.

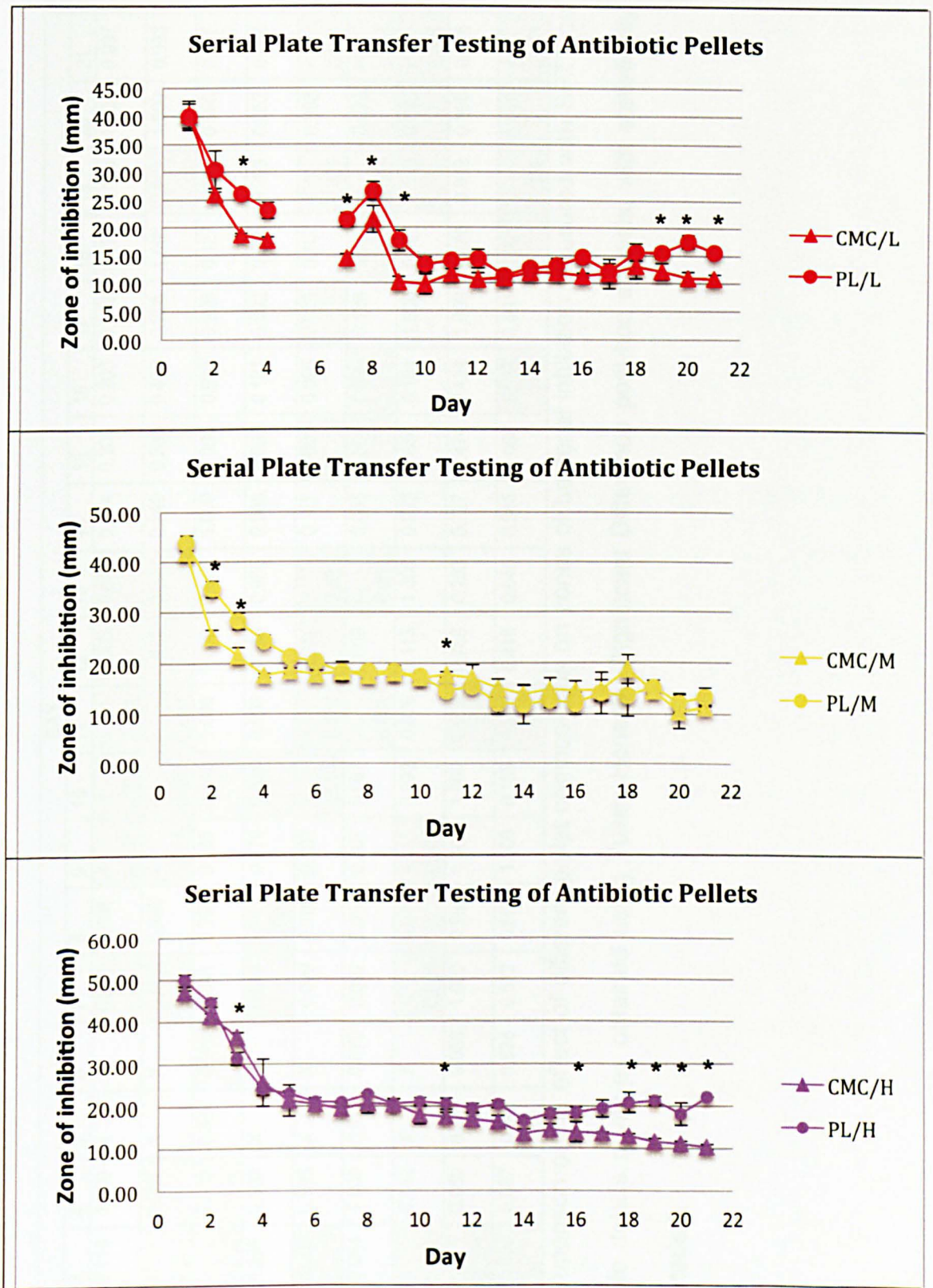


Fig 11.3.3.1d/e/f: SPTT: Effect of carrier in pellets with low (d, top), medium (e, middle) and high (f, bottom) drug loading. Error bars indicate Standard Deviation, and stars the day on which the different levels were statistically significant.

Serial Plate Transfer Testing

	DAY																			
GROUP	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
CMC/L CMC/M	0.927	0.995	0.118	1.000	X	X	0.011	0.095	< 0.001	< 0.001	< 0.001	0.003	0.047	0.744	0.207	0.203	0.678	0.124	0.186	1.000	0.997
CMC/L CMC/H	0.007	< 0.001	< 0.001	0.017	X	X	0.002	0.996	< 0.001	< 0.001	< 0.001	0.004	0.008	0.849	0.289	0.400	0.908	1.000	0.001	1.000	0.993
CMC/M CMC/H	0.034	< 0.001	< 0.001	0.018	0.333	0.043	0.841	0.200	0.285	0.047	1.000	1.000	0.885	1.000	1.000	0.995	0.996	0.127	0.075	0.996	0.915
PL/L PL/M	0.149	0.118	0.295	0.990	X	X	0.049	< 0.001	0.978	0.035	0.993	0.995	0.985	0.989	0.995	0.181	0.092	0.905	0.999	0.052	0.060
PL/L PL/H	< 0.001	< 0.001	0.002	0.995	X	X	0.999	0.109	0.107	< 0.001	< 0.001	0.017	< 0.001	0.147	0.009	0.095	0.023	0.167	< 0.001	0.998	< 0.001
PL/M PL/H	0.012	< 0.001	0.084	1.000	0.610	0.670	0.092	0.051	0.307	0.067	< 0.001	0.069	< 0.001	0.056	0.004	0.003	0.126	0.032	< 0.001	0.026	< 0.001
CMC/L PL/L	0.999	0.056	< 0.001	0.142	X	X	< 0.001	0.019	< 0.001	0.092	0.076	0.113	1.000	0.992	0.864	0.181	1.000	0.792	0.033	0.015	< 0.001
CMC/M PL/M	0.718	< 0.001	< 0.001	0.060	0.389	0.068	1.000	0.983	1.000	1.000	0.010	0.788	0.202	0.727	0.474	0.424	1.000	0.188	0.972	0.898	0.088
CMC/H PL/H	0.371	0.307	0.003	0.962	0.682	0.838	0.572	0.663	1.000	0.168	0.017	0.418	0.040	0.345	0.060	0.037	0.091	0.021	< 0.001	0.010	< 0.001

Table 11.3.3.1: Comparison of the effect of different pellet compositions on zones of bacterial inhibition measured with SPTT on different days. Table details p values obtained using Tukey Honestly Significant Difference post-hoc analysis, with statistically significant values shaded.

In addition to studying zones on individual days, the total sum of the zones obtained on all days by the different pellets was also compared (results detailed below).

Effect of antibiotic loading

Increasing antibiotic loading led to increased zones of inhibition; however, this was seen only during the first 13 days in the case of CMC pellets, although the effect was still evident at 21 days in the case of pluronic pellets. The total sum of zones obtained by CMC/L pellets (284.9 mm) was significantly lower than the sum of those seen in CMC/M (375.9 mm, $p=0.001$) and CMC/H pellets (424.0 mm, $p<0.001$), although CMC/M and CMC/H pellets did not differ significantly ($p=0.061$). PL/H pellet total zone (496.3mm) was significantly larger than the zones obtained by PL/M (391.1mm, $p<0.001$) and PL/L (354.9mm, $p<0.001$), although PL/L did not differ significantly from PL/M ($p=0.217$)

Effect of carrier gel

Pluronic generally gave larger zones of inhibition than CMC, seen both in the early days but particularly in the later days of the experiment. The total sum of zones in the case of low and high antibiotic loading was higher with pluronic pellets than CMC ($p=0.005$ and 0.004, respectively), but the difference was not statistically significant in medium antibiotic concentrations ($p=0.902$).

11.3.4 Pellet weight and diameter

To assess pellet swelling and water uptake during experiments, the pellet diameter was measured when performing SPTT, and the pellets weighed when changing the PBS solution for HPLC. Pellet weights and diameters are shown in Fig 11.3.4.1, showing all pellets first (a), followed by comparison of carrier choice whilst keeping drug level constant (b, c, d). Statistical significance is indicated by a star on graphs b-d.

Pellets containing pluronic tended to swell less and gain less weight (indicating less water uptake) than those made with CMC. This was also obvious by observation as the pluronic pellets kept their shape better.

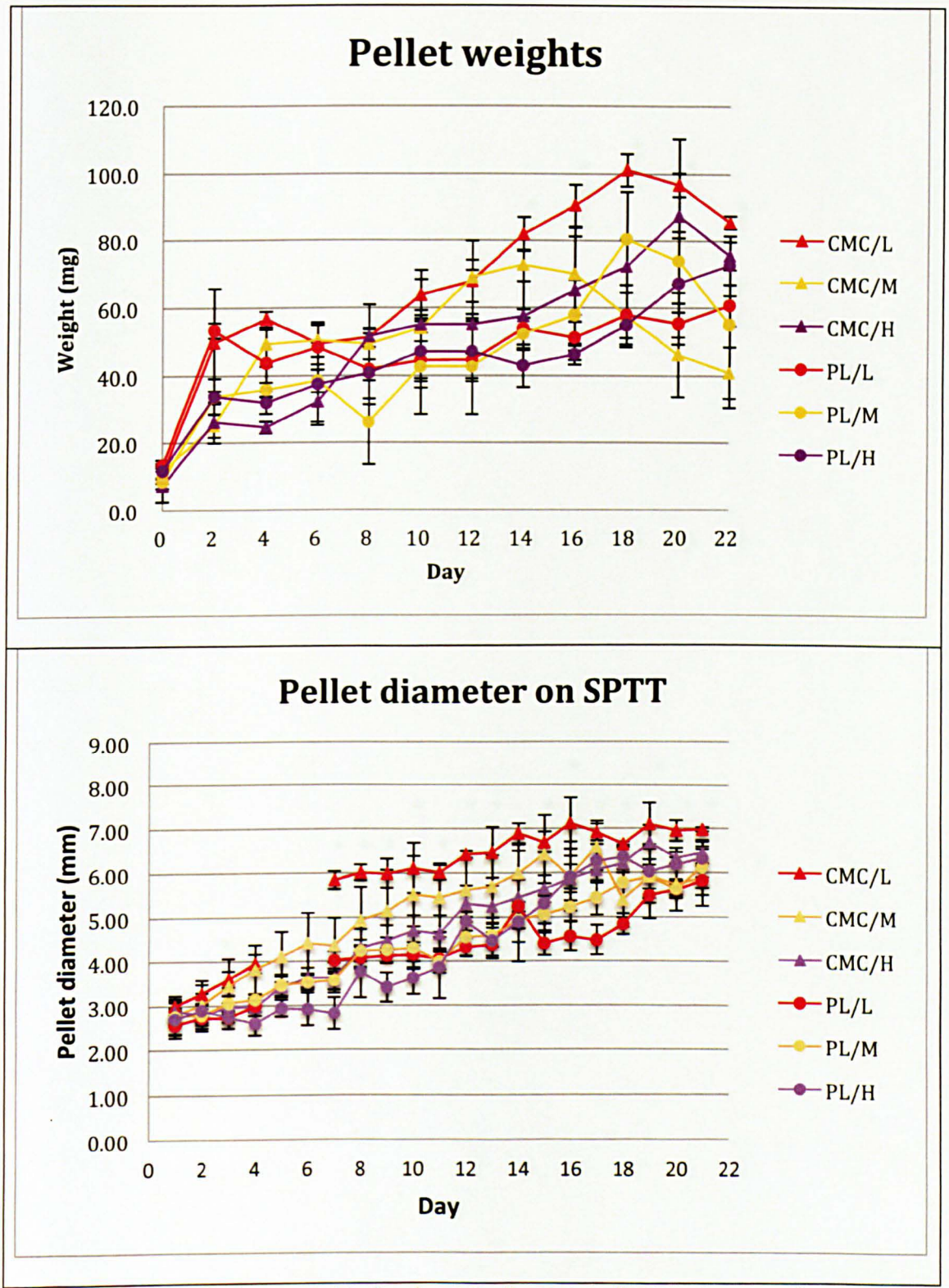


Fig 11.3.4.1a: Pellet weight (top) and diameter (bottom) over time. Pellet composition is shown in Table 11.1.1. Error bars indicate Standard Deviation.

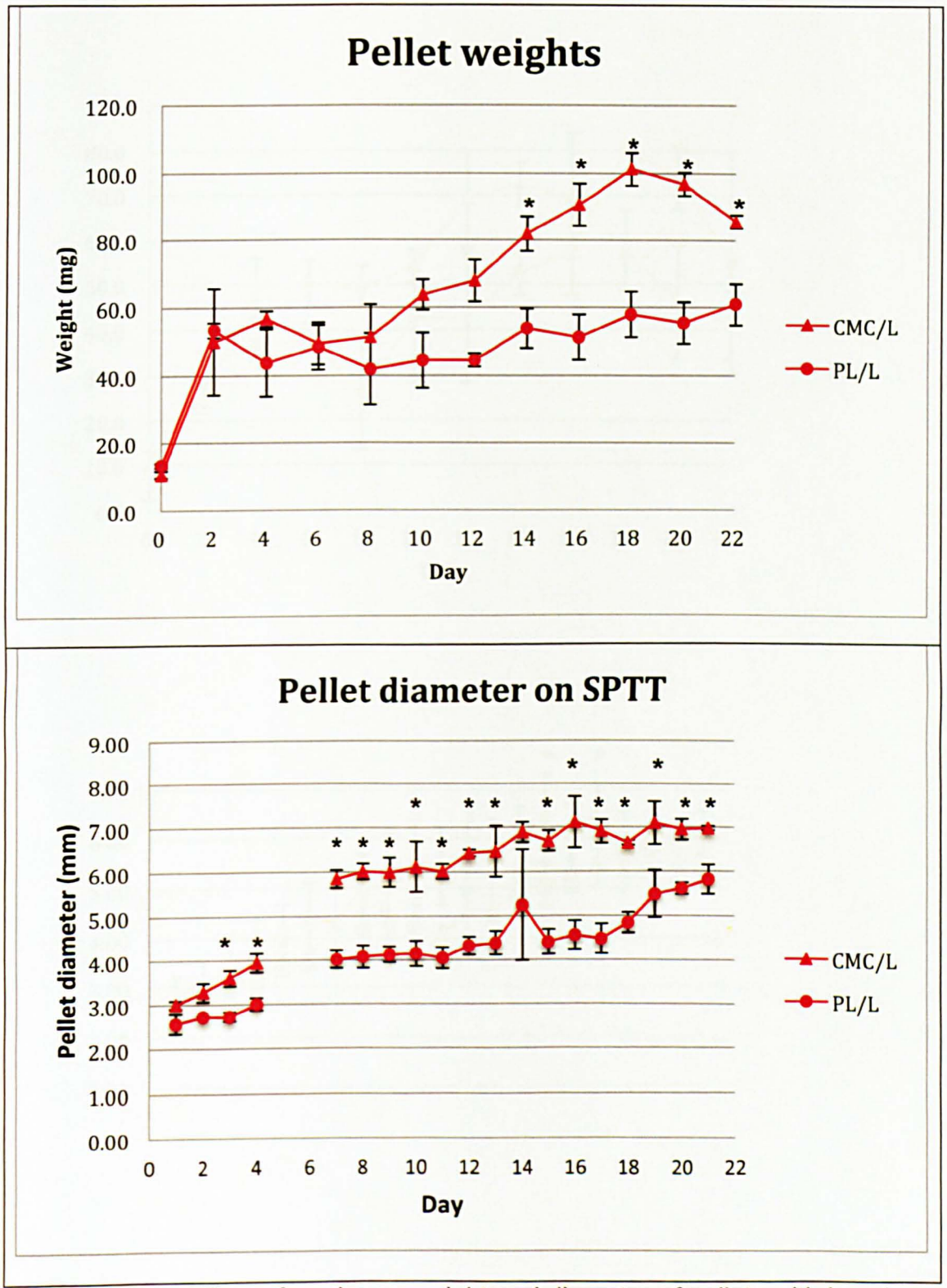


Fig 11.3.4.1b: Effect of carrier on weight and diameter of pellets with low drug loading over time. Error bars indicate Standard Deviation, and stars the day on which the different weights / diameters were significantly different.

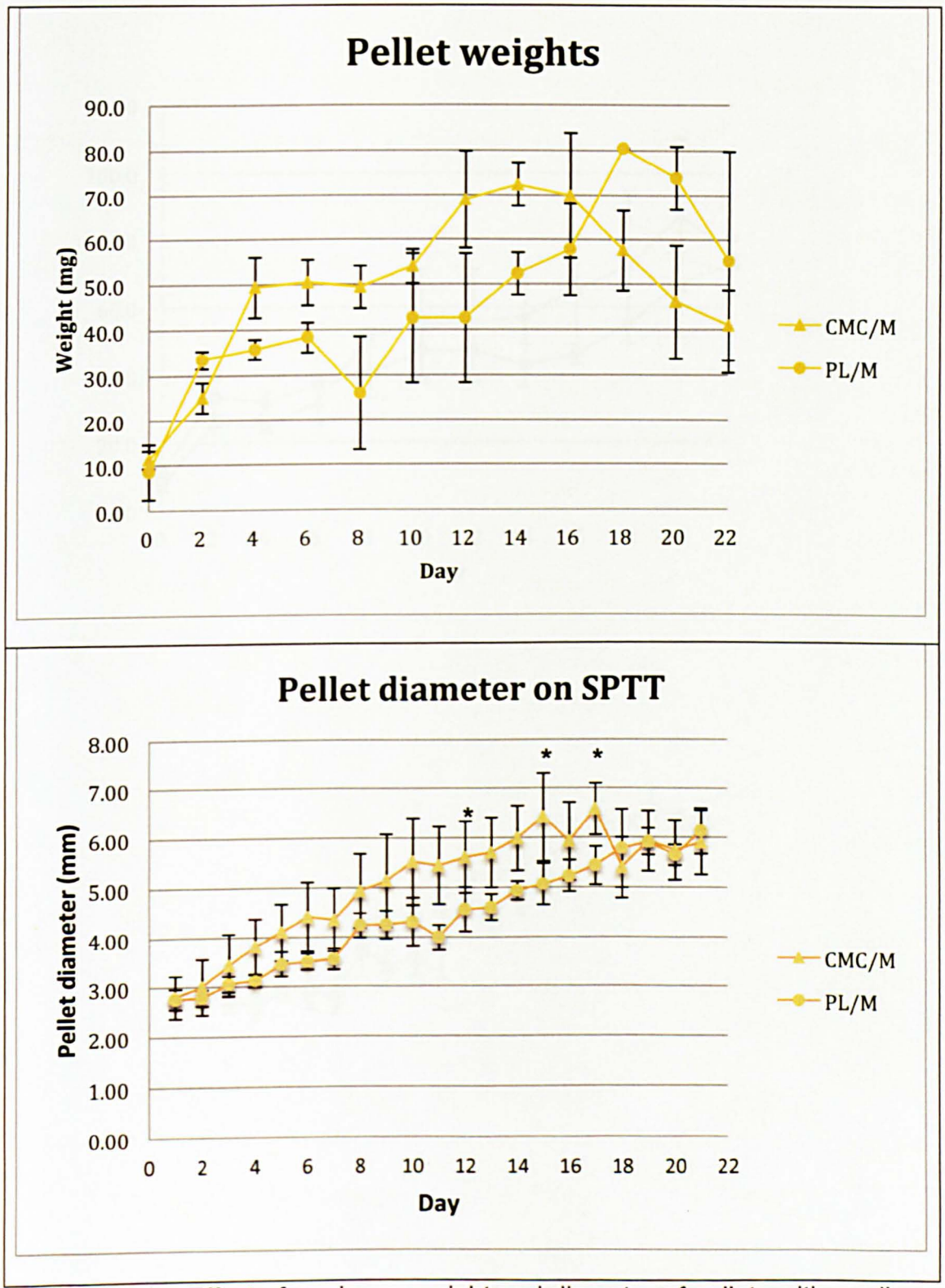


Fig 11.3.4.1c: Effect of carrier on weight and diameter of pellets with medium drug loading over time. Error bars indicate Standard Deviation, and stars the day on which the different weights / diameters were significantly different.

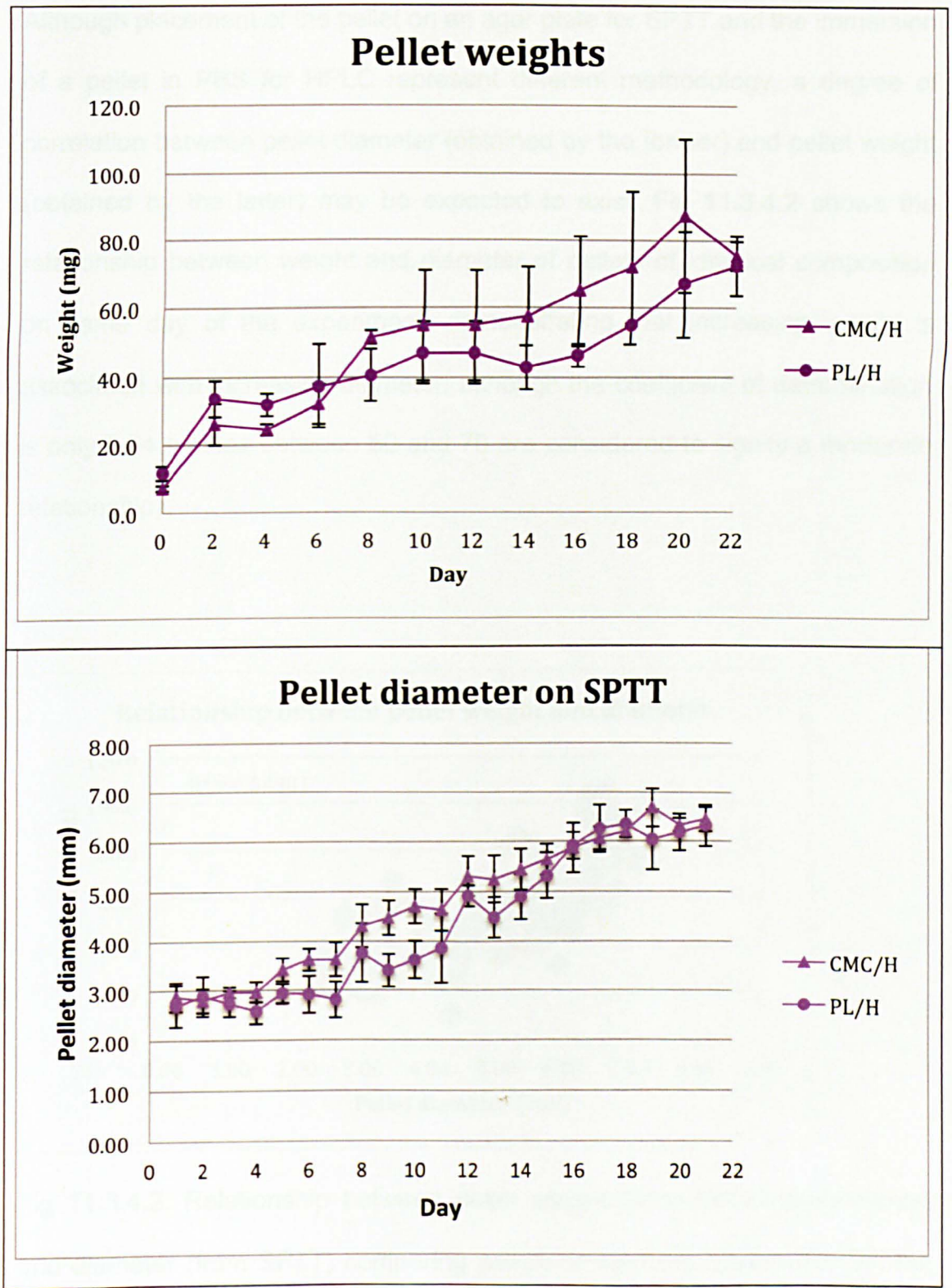


Fig 11.3.4.1d: Effect of carrier on weight and diameter of pellets with high drug loading over time. Error bars indicate Standard Deviation. Weights / diameters were not significantly different on any day.

Although placement of the pellet on an agar plate for SPTT and the immersion of a pellet in PBS for HPLC represent different methodology, a degree of correlation between pellet diameter (obtained by the former) and pellet weight (obtained by the latter) may be expected to exist. Fig 11.3.4.2 shows the relationship between weight and diameter of pellets of identical composition on same day of the experiment, demonstrating that increasing weight is associated with increasing diameter, although the coefficient of determination is only 0.54 (values between 50 and 70 are considered to signify a moderate relationship).

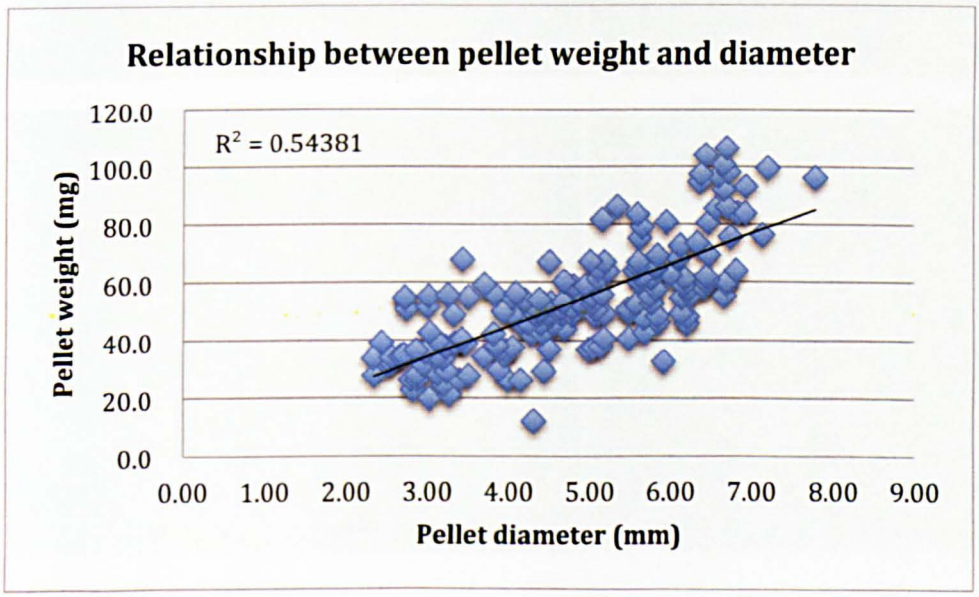


Fig 11.3.4.2: Relationship between pellet weight (from HPLC experiments) and diameter (from SPTT) comparing pellets of identical composition on the same experiment day.

11.3.5 Biofilms on pellets

Following 21 days of SPTT, the inactive pellets with no antibiotics were examined with SEM to see if biofilms were growing on them (Fig 11.3.5.1). The pellet surface was noted to be completely covered with a biofilm, which in many cases could also be seen without the aid of magnification.

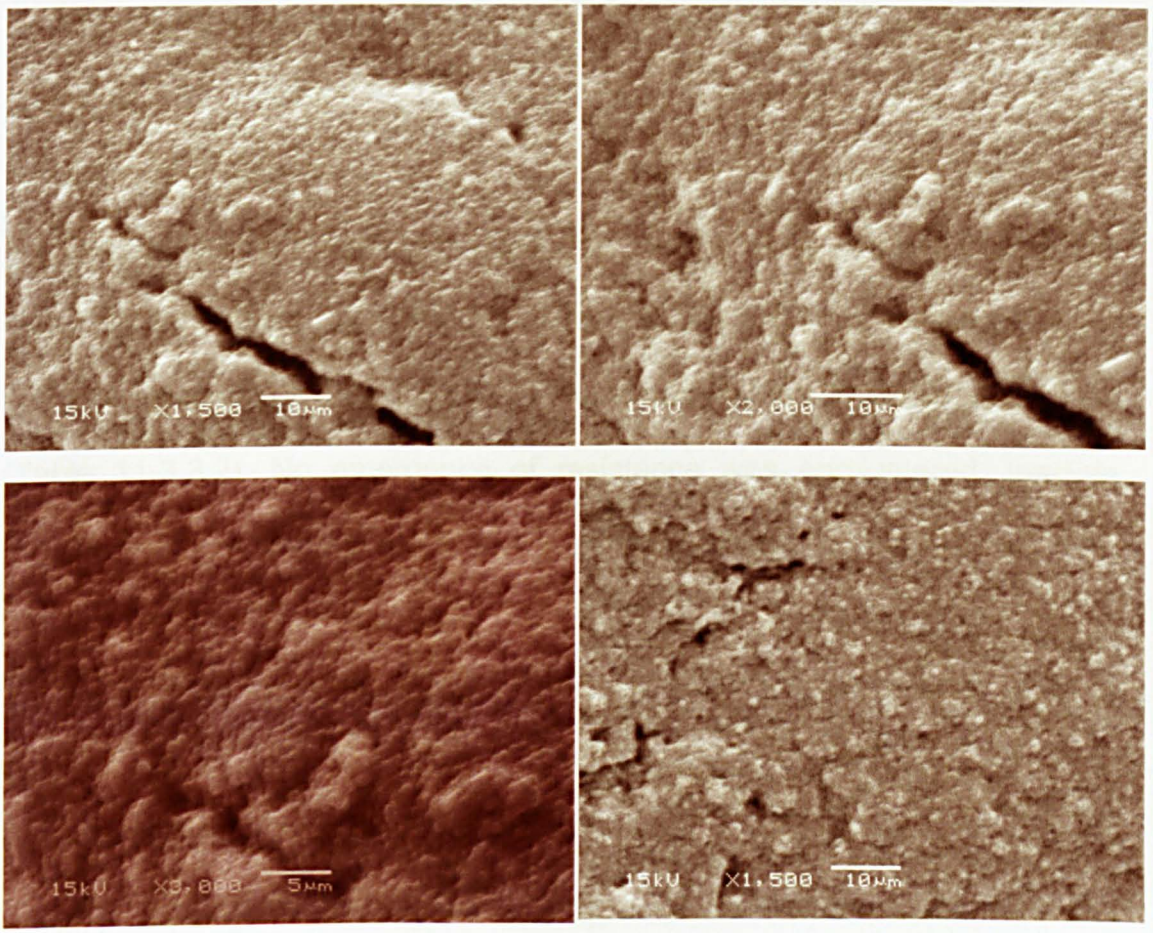


Fig 11.3.5.1: Bacterial biofilms (*S aureus* F2315) on inactive pellets after 21 days of SPTT (Jeol 6060LV variable pressure SEM). The surface is completely covered with bacteria.

11.3.6 Effectiveness of antibiotic pellets in the *in vitro* biofilm model

Five day old biofilms of *S aureus* were used to test effectiveness of the antibiotic pellets against biofilms in the *in vitro* model described in Chapter 8. The biofilms were exposed to antibiotic pellets for 21 days, during which time TSB was changed every 2 days to remove any antibiotic already released by the pellets. At the end of the 21 days, the antibiotic pellets were removed and biofilms placed in fresh TSB to allow a 5 day period of "resuscitation" prior to sampling.

Irrespective of the carrier gel used, the pellets made with low antibiotic concentrations failed to eradicate biofilms, but the pellets containing medium and high concentrations successfully eradicated biofilms in the *in vitro* model (Table 11.3.3.2). No emergence of resistance was encountered. Inactive pellets without antibiotics had no anti-biofilm properties.

PELLET	BIOFILM ERADICATED?
CMC/L	No
CMC/M	Yes
CMC/H	Yes
PL/L	No
PL/M	Yes
PL/H	Yes

Table 11.3.3.2: Antibiotic pellets and biofilm eradication in the *S aureus in vitro* biofilm model.

11.4 DISCUSSION

11.4.1 Summary of results

Drug release from antibiotic pellets was assessed in two complementary ways: using High Performance Liquid Chromatography (following development of a new method that allowed simultaneous detection of rifampicin and clindamycin) and with Serial Plate Transfer Testing. HPLC showed that increasing clindamycin loading of the pellets led to greater antibiotic release, although this was confined to the early days, and pellets containing pluronic gave higher antibiotic levels in later days than those containing CMC. Increasing rifampicin loading also led to higher drug levels released on days 2 and 4, but the rifampicin levels detected with HPLC beyond day 4 were too low to allow meaningful interpretation. SPTT showed that addition of increased antibiotic concentrations led to greater zones of bacterial inhibition well into the second week in the case of CMC and right to the end of the three week experiment in the case of pluronic. Pellets made with pluronic also gave consistently larger inhibition zones than those made with CMC, and additionally they swelled less. The antibiotic pellets were also tested in the newly developed *in vitro* biofilm model, and the pellets containing the two higher antibiotic loading levels were found to be effective at eradicating biofilms.

11.4.2 High performance liquid chromatography

HPLC is a technique used for the separation of mixtures. The HPLC column consists of tightly packed particles (stationary phase), through which a high-

pressure pump pushes the solution to be analysed mixed with a liquid mobile phase. Different molecules will be slowed down by their passage through the column by different amounts, thus allowing separation of different compounds by time, with each substance having a characteristic retention time for a given mobile and stationary phase. Once the substance has passed through the column, it is detected using an ultraviolet detector.

We employed reverse-phase chromatography, which consists of a non-polar stationary phase; the term reverse-phase is historic, because older HPLC techniques employed a polar stationary phase, but reverse-phase chromatography is now the most widely used method. Reversed-phase chromatography operates on the principle of hydrophobic forces. Polar substances bind to the column less and have shorter retention times. Non-polar substances bind to the column more and have a longer retention time; however, as the mobile phase is made more hydrophobic, a point is reached where the analyte moves preferentially into the mobile phase and is eluted (this variation in mobile phase during the run itself is called gradient elution, as opposed to the isocratic method where the composition of mobile phase is kept constant). Thus the binding of the analyte to the non-polar particles in the column depends on the degree of polarity of the analyte, which is affected by the degree to which it is ionised, and this in turn depends on the pH of the mobile phase in relation to the properties of the analyte.

The HPLC column used in our experiments is typical of columns used in many HPLC applications, as is the use of a mobile phase composed of methanol,

water and acetonitrile; however, in order to measure rifampicin and clindamycin release from the pellets used in this study a new HPLC method had to be developed. HPLC has been used to measure rifampicin (Koga 1987) (Zhou, et al. 2010) and clindamycin (Koga 1987) (La Follette, et al. 1988) before, but to the best of our knowledge this is the first time that the two antibiotics have been measured by HPLC simultaneously; of course, one could have measured them separately by processing each sample twice with HPLC using different methods, but this would require more time and resources, and may be difficult with small volume samples.

The chromatograms do not give antibiotic concentration as the output but give the light absorption at the chosen wavelength. The area under the peak is related to antibiotic concentration; therefore, these two variables can be used to plot a standard line, so that peak area can then be used to calculate antibiotic level. It is important to ensure that the standard line is an accurate fit, as this ensures accuracy of subsequent calculations. The R^2 coefficient of determination describes how well the two variables relate to each other, as it describes what proportion of variability in a data set is accounted for by the statistical model, with a value closest to 1 implying excellent relationship; in these experiments the R^2 for clindamycin was 0.998 and for rifampicin 0.999, indicating excellent correlation.

Both antibiotics can still be detected down to 2 µg/mL with this method. In the case of rifampicin this equates to about 333 times the minimum inhibitory concentration of the test *S aureus* strain, and in the case of clindamycin to 20

times. The experiments focusing on biofilm eradication would be likely to require antibiotic levels in the region of 1000 to 10,000 x MIC (rifampicin 6 – 60 mg/L and clindamycin 100 – 1000 mg/L) based on our previous experiments (Chapter 9) and published data (Ceri, et al. 1999) (Donlan and Costerton 2002) (Nickel, et al. 1985) (Olson, et al. 2002) (Vorachit, et al. 1993). Therefore, the detection limits of this HPLC method are acceptable when measuring antibiotic levels in the context of biofilm eradication. If one needed to detect lower levels then the samples could have been concentrated prior to analysis and the value then adjusted accordingly.

Alternatives to HPLC (Catena, et al. 2009) include determination of antibiotic concentrations by spectrophotometry, but as the absorption spectra of the two antibiotics overlapped this technique would not be suitable. Mass spectrometry could be used although it introduces more complexity into the methodology. Microbiological or immuno-based assays could have been developed as an alternative.

11.4.3 Choice of modified-release device

The application of novel drug delivery for the local treatment of infection has received much attention, particularly in relation to implantable materials and surface coating (Bayston, Ashraf and Bhundia 2004) (Bayston, Fisher and Weber 2009) (Furno, et al. 2004). Biodegradable polymers have been identified as a potential method for delivering antibiotics in a modified-release manner (Smith 2005), and in addition to surface coating or impregnation other strategies to release antibiotics into the surrounding milieu have also been

examined. For example, in the field of ocular drug delivery the duration of antibiotic release has been shown to be possible in excess of 4 weeks, although not necessarily at anti-biofilm concentrations (Anderson, et al. 2009) (Garty, et al. 2011). Another study of a matrix PLGA / penicillin formulation to deliver antibiotics to the pleural cavity achieved drug release for up to 30 days; following the initial high amount of drug released, subsequent lower levels were still above the minimum inhibitory concentration of common organisms for 2 weeks *in vivo* and 4 weeks *in vitro* (Liu, et al. 2011). When treating biofilms it is important that sufficiently high antibiotic levels are reached, i.e. above minimum biofilm eradication concentration rather than just above the minimum inhibitory concentration of planktonic bacteria, otherwise the mutant selection window is entered and resistance may emerge.

Within the middle ear, studies using modified drug delivery to eradicate middle ear infections have also been published. Goycoolea et al (Goycoolea and Muchow 1994) (M. Goycoolea, D. Muchow, et al. 1991) (M. Goycoolea, D. Muchow, et al. 1992) studied modified-release ampicillin from a poly-L-lactic acid support in cat and chinchilla animal models, and found that the method was effective at eradicating infection without any ototoxicity. Whilst encouraging, this is not a true representation of human OME, as animal models were either acute infections or Eustachian tube obstructions and no true animal model of OME exists. In addition, the important link to biofilms and their eradication was also not explored.

Another ear study using pluronic F-127 to deliver vancomycin as a treatment of experimentally induced MRSA middle ear infection (Le, et al. 2004) is again encouraging but not a true representation of human OME. Antibiotic-impregnated ventilation tubes also been investigated with the intention of preventing colonisation or even releasing drugs (ciprofloxacin and usnic acid) into the middle ear effusion, but the antibiotic levels released appear to be low (Labib, et al. 2009).

PLGA is one of the most commonly used biodegradable systems (Bossy, et al. 2008), with a long history of clinical use in sutures, implants, prosthetic devices, and drug delivery systems. PLGA is generally considered to be very safe (Anderson and Kim 1984), making it an ideal starting point for developing new treatments for OME. The different types of PLGA have different molecular weight, different ratios of lactic to glycolic acid, and can be made as differently-sized microparticles; all these characteristics affect the rate of degradation (Bossy, et al. 2008). For our purposes 56 kDa PLGA 50:50 with mean particle size 12 μm was suitable as a starting point.

The PLGA microparticles adhere to each other when heated (sintered) to form a porous matrix in the desired shape. The spaces in the matrix between PLGA microparticles are filled by a gel that contains the antibiotics. The modified-release properties of the device are thus conferred both by the gel containing the antibiotics and the PLGA matrix. The gel releases the antibiotics at a slow rate depending on water ingress and concentration gradients, whilst the PLGA matrix governs water ingress through the pores,

and PLGA degradation over time increases release of gel and drug from within the matrix. PLGA undergoes bulk erosion, so that at the end of its life the device simply disintegrates into small fragments; PLGA undergoes hydrolysis to lactic and glycolic acid, both of which are metabolised in man without adverse reaction.

CMC and poloxamer are hydrogels with modified-release properties. Hydrogels have been widely studied for their modified-release properties (Anderson and Kim 1984) (Simoes, Figueiras and Veiga 2012). They are polymeric materials consisting of hydrophilic macromolecules cross-linked to form a three-dimensional network, and have the ability to imbibe large quantities of water without dissolving. Drug molecules can then be dispersed or dissolved within the hydrogel and its retained water, and are released through diffusion.

CMC is a cellulose derivative with modified-release applications, and is widely used as a food viscosity modifier, is a component of many tooth pastes, and forms the outer gel lining of nasal packing or wound dressings (Lim, et al. 2010). CMC is available in a range of viscosities, with the high viscosity CMC chosen in these studies known to lead to more prolonged drug release (Emeje, Kunle and Ofoefule 2006). Although generally considered safe (Dumond, et al. 2009) (Shen, et al. 2011), one animal study of its use as ear packing material suggested that it might affect hearing (Antonelli, Sampson and Lang 2010), but another found no concerns (Jang, et al. 2008). Although CMC can be degraded by cellulase this enzyme is not present in man, and

the gel (whether CMC or poloxamer) may therefore be dissipated down the Eustachian tube once the PLGA degrades.

Poloxamers, also known by their trade name pluronics, are triblock co-polymers, consisting of a central hydrophobic polyoxypropylene chain flanked by two hydrophilic polyoxyethylene chains. Variation in the length of the polymer chain and exact percentage polyoxyethylene content lead to variation in the molecular weight, physical form, and drug release properties. In addition to being used in modified drug release (Chae, Mo and Oh 2010) (Manjunath, Venkateswarlu and Hussain 2011), poloxamers are also mucoadhesive (Dumortier, et al. 2006), and have thermo-reversible properties which means that they can be injected into the body as a liquid that turns into gel at body temperature (Wang, Dellamary, et al. 2009) (Wang, Dellamary, et al. 2011). Poloxamers are widely used in cosmetics and mouthwashes, and generally considered safe (Singh-Joy and McLain 2008), with animal experiments showing no ototoxicity and only temporary hearing impairment likely due to conductive loss caused by gel in the middle ear (H. Feng, et al. 2007) (Feng, Sun and Jiang 2008) (Wang, Dellamary, et al. 2009) (Wang, Dellamary, et al. 2011). Poloxamers are not biodegradable but are rapidly excreted in the urine following systemic administration (Singh-Joy and McLain 2008), and the gel released from the pellet my be absorbed or dissipated down the Eustachian tube once the PLGA device degrades.

11.4.4 Antibiotic release

The type of a modified-release delivery device studied in this research, a matrix system with drug dispersed throughout the polymer matrix and gel, has several advantages over other types as discussed in Chapter 10 (Anderson and Kim 1984), most notably the ease with which drug delivery rate, dosing, lifetime of the device, and its size / shape can be adjusted. However, a disadvantage is the difficulty of achieving drug release at a constant level, with the typical release profile being of a burst followed by gradually reducing levels (Anderson and Kim 1984). Hydrogels may also have a relatively rapid drug release profile, and have limited capacity to hold hydrophobic drugs (Simoes, Figueiras and Veiga 2012).

The pellets studied here are typical of a matrix device, as increasing drug loading of the pellets increased the amount of drug released, and the release profile is one of an initial burst followed by release at much lower levels. SPTT and HPLC give complementary information about drug release, with the former assessing both antibiotics together with the pellet sitting on a moist agar plate, and the latter measuring levels of antibiotics separately with the pellet immersed in saline. In terms of antibiotics diffusing out of the pellet, having the pellet sitting on a moist agar plate is more likely to be representative of the middle ear conditions following VT insertion. Having the pellet fully immersed in PBS (changed every 2 days) for HPLC experiments is likely to represent extreme conditions designed to provide maximum diffusion gradient and pellet wetting. In fact, PBS is known to have a stronger effect on physical and mechanical properties of PLGA scaffolds than water or tissue

culture medium (Perron, et al. 2009), and the drug release profile in PBS is different than *in vivo*, with PBS leading to a greater burst release (Liu, et al. 2011). Other characteristics of the surrounding milieu, such as pH and temperature, also influence drug release (Faisanta, et al. 2006).

SPTT experiments showed a much less steep fall in the amount of drug release (using zones of bacterial inhibition) than HPLC, with continued drug release for 3 weeks. However, HPLC showed that only clindamycin was being released at measurable levels beyond day 4, and because SPTT assesses both antibiotics together no information can be drawn about clindamycin / rifampicin differentials on SPTT. Although the lower rifampicin levels released may relate to lower rifampicin loading into the device (chosen to reflect differential susceptibility of test organism to the two antibiotics), this may also be due to poorer rifampicin water-solubility: the hydrogel imbibes a large quantity of water containing the drug, so if a drug is more hydrophobic the quantity of the drug that can be incorporated into the hydrogel is limited, and drug release tends to be fast (Simoes, Figueiras and Veiga 2012).

The downside of achieving therapeutic concentrations for only one antibiotic is that emergence of resistance is more likely than when multiple antibiotics are used (D'Agata, et al. 2008) (Olofsson and Cars 2007). The actual antibiotic concentrations released are also important, because levels below minimum biofilm eradication concentration would favour emergence of resistant strains without eradicating the biofilm itself, or they may promote biofilm formation (Kaplan, Antibiotic-induced biofilm formation 2011). Assuming that the

minimum biofilm eradication concentration level is 1000 times higher than minimum inhibitory concentration of planktonic bacteria, then the pellets studied here can achieve those levels only on Day 2 as tested by HPLC, but not on subsequent days. Given that pellets without antibiotics were noted to have biofilms growing on their surface, the pellet might actually act as a focus of infection and biofilm attachment itself, particularly if antibiotic levels were low (Campoccia, et al. 2010).

The pellets that were sintered for a shorter time, or contained medium viscosity CMC, or a ratio of PLGA to carrier gel of 1:0.6 instead of 1:0.8, were not as well formed as the pellets made with high viscosity CMC and sintered for 16 hours, and were noted to disintegrate early. Although the amount of drug released may be increased, this occurs at the expense of earlier pellet degradation, and is therefore not suitable if one requires the pellet to remain intact for approximately 3 weeks to prevent smaller disintegrating particles moving down the Eustachian tube. Of course, a variety of different permutations of carrier gel type, concentration, and processing variation exist, although these have not been investigated further here.

Overall, the results indicate that the 20% pluronic F127 gel used in these experiments would be a better choice than 2% high viscosity CMC, as drug is released in larger quantities for a longer time period. If antibiotic loading is kept constant, pellets made with pluronic achieved higher clindamycin release on days 18 and 20 as measured by HPLC. Due to low rifampicin levels measured by HPLC beyond day 4 no comment can be made to compare

pluronic with CMC. Pluronic pellets consistently gave higher zones of inhibition than CMC pellets when examined with SPTT. The pluronic pellets also swell less, an important factor when placing a pellet into the constrained middle ear space.

The phenomenon of system saturation (Adams, et al. 2009) is also well seen when clindamycin is measured with HPLC. Above a certain drug level the modified release system cannot hold any more drug, and any drug above this saturation point is simply released straight away. This is seen as a higher percentage of drug being released in the first few days from those pellets with high loading, and the achievement of higher antibiotic levels. However, no real difference in antibiotic levels achieved beyond the first few days occurs when pellets with high loading are compared with those with lower antibiotic loading.

11.4.5 Biofilm eradication

The drug levels released from the antibiotic pellets as measured by HPLC would not be expected to eradicate biofilms, based on the high antibiotic levels required (Chapter 9) (Ceri, et al. 1999) (Donlan and Costerton 2002) (Nickel, et al. 1985) (Olson, et al. 2002) (Vorachit, et al. 1993). Although the pellets were effective against bacteria on SPTT, this tests inhibitory activity against bacteria in colonies grown on an agar plate, which resemble planktonic bacteria and not biofilms (Mikkelsen, et al. 2007). However, despite concerns that antibiotic levels may be lower than minimum biofilm eradication concentration beyond day 2, and that only one antibiotic rather than two may be released, the pellets containing the two higher antibiotic levels investigated

nevertheless managed to eradicate *S aureus* biofilm grown in an *in vitro* model. The lowest concentration studied did not achieve this, but no emergence of resistance was encountered.

Clearly the high initial burst of antibiotics, a useful anti-biofilm strategy, was sufficient for biofilm eradication despite lower antibiotic levels on subsequent days. This apparent discrepancy with results presented in Chapter 9 may be explained by the fact that antibiotic concentration was kept constant in Chapter 9, but varied in the case of release from pellets. It is possible that a high initial concentration, followed by a lower level is adequate for biofilm eradication, indeed the experiments using pellets to eradicate biofilms suggests this is so. The experiments in Chapter 9 thus do not exactly replicate conditions when pellets are used, but the success of pellets is encouraging because eradication could be achieved even in the absence of zero order kinetics. A published study of modified release levofloxacin against *E coli* biofilms also showed that a formulation that gave high burst release but subsequent lower levels was more effective than an alternative that had a lesser burst but higher antibiotic levels beyond day 1 (Cheow, Chang and Hadinoto 2010).

The effectiveness of the pellet in the *in vitro* model also supports the idea of local drug delivery as a means of optimising the therapeutic window: achieving at the site of infection antibiotic levels much higher than could be obtained with systemic administration, whilst minimising any systemic toxicity.

11.4.6 Further investigations

Although promising, the formulation that we have investigated to date has a number of shortcomings. Although antibiotics can be released for up to 3 weeks, the actual levels obtained beyond the first few days are low, and only clindamycin, not rifampicin, is released at any significant level after day 4. Whilst preliminary work is encouraging, further research is needed to ensure the desired objective of releasing all antibiotics for a period of several weeks, before the pellets would be suitable for clinical use.

Additionally, the formulation has so far been tested only against *S aureus*, but patient use would require demonstration of its effectiveness against other bacteria (for example *Streptococcus pneumoniae, Haemophilus influenza, Moraxella catarrhalis*), with incorporation of additional antibiotics, for example amoxicillin; macrolides may also be beneficial as they have biofilm-inhibiting, immunomodulating and inflammation-resolving activity (Perletti, et al. 2011). It would also be useful to explore diffusion of antibiotics through the middle ear effusion. The experiments undertaken to date were performed *in vitro*, but future *in vivo* testing may also be appropriate, both in terms of toxicity and efficacy; although there is no exact animal model of OME, the existing approximations may nevertheless be of value in research on antibiotic pellets for OME biofilms (Hardisty-Hughes, et al. 2006) (Parkinson, et al. 2006).

Given that the antibiotic levels required for biofilm eradication are high in relation to the size of the antibiotic pellet that can fit into the small middle ear space, any strategy that would minimise antibiotic levels required for biofilm

eradication would be welcome. Therefore, future work would also examine the addition of N-acetyl cysteine (NAC) to antibiotics as a means of reducing the amount of antibiotic required to eradicate biofilms. NAC is a mucolytic with established anti-biofilm properties and an excellent safety profile (Aslam et al 2007) (Aslam, Trautner, et al. 2007) (Olofsson and Cars 2007). It is thought to decrease biofilm formation by reducing the production of extracellular polysaccharide matrix (Ovesen, et al. 2000) while promoting the disruption of mature biofilm. Several groups, including ours (Al-Zahid, et al. 2011), have shown that NAC has a synergistic effect when used with antibiotics. NAC has also been used as an adjunct to grommet insertion by being applied directly to the middle ear at the time of grommet insertion (Ovesen, et al. 2000); it has been shown to reduce, but not eliminate, the need for repeat surgery. If NAC alone cannot lower the antibiotic levels required for biofilm eradication sufficiently to allow our antibiotic strategy to work, alternatives such as the use of DNase (Kaplan, et al. 2012) to potentiate antibiotic action could be explored, as may quorum sensing inhibitors.

A number of ways of prolonging release from monolithic devices are available (Anderson and Kim 1984), as discussed in Chapter 10, including reducing ingress of water for example with the addition of an impervious surface barrier. Additionally, rather than using a monolithic device, an alternative modified-release technology such as encapsulation could be utilised (Joung, et al. 2007); the size of PLGA spheres and their molecular weight can be manipulated to achieve the desired release profile, with smaller spheres releasing drugs more quickly largely due to their relatively greater surface

area (Li and Jasti 2006), and lower molecular weight PLGA results in more porous formulation and therefore faster drug release. Chitosan has also been identified as a natural cationic polymer that strongly binds anionic antibiotics (such as rifampicin) and with drug release potentially exceeding 30 days (Cao and Sun 2009) (Stinner, et al. 2010).

Drugs delivered to a mucosal surface should ideally adhere to the mucosa, as increased contact time will improve drug delivery (Li and Jasti 2006). The mucus coats many epithelial surfaces, is released from mucus-secreting glands and goblet cells, and serves as a lubricant or a protection barrier (Serra, Domenech and Peppas 2009). The typical turnover time is in the region of 6 hours. Although mucus consists mainly of water, the presence of mucins (glycoproteins consisting of a protein core with branched oligosaccharide chains) gives mucus its characteristic gel-like property. Muco-adhesion, the attachment of polymers to a mucosal surface, is not well understood (Serra, Domenech and Peppas 2009), but is thought to involve wetting and swelling of polymer to allow close contact with human tissue, interdigitation and entanglement of polymer and mucin chains, and formation of weak chemical bonds. However, muco-adhesive techniques achieve a stay time usually in the region of several hours (Tao, et al. 2009), and are thus not applicable to the design of a device for middle ear that is required for several weeks. Rather, the middle ear implant will rely on its size for its stay time, although muco-adhesion could nevertheless be a useful characteristic.

A further future refinement could include stimulus-responsive drug delivery that could release antibiotics in response to the presence of an active infection. Stimulus-responsive drug delivery represents a further advance in modified drug delivery (Saltzman 2001) (Uchegbu and Schatzlein 2006). These devices release drug in response to a predetermined stimulus, such as a change in temperature, pH, ionic strength, or other external stimuli e.g. light, electric current or magnets or ultrasound (Bajpai, et al. 2010) (Morishita and Park 2010). Thermo sensitive and pH-sensitive systems represent a fine balance between hydrophobic and hydrophilic structure, where a small change in the environment tips the balance from one state to the other, resulting in drug release (Bajpai, et al. 2010).

11.4.7 Clinical applications

Whilst further developments are required, a novel strategy based on modified-release antibiotic pellets for the treatment of OME biofilms may help improve care of patients in the future. The pellet could be inserted into the middle ear at the time of VT insertion with the aim of reducing the need for revision surgery. Alternatively, the pellet may be useful on its own, inserted in combination with just a myringotomy (or perhaps using a thermo-sensitive injectable formulation rather than a pellet), thus avoiding VT insertion and their potential complications (including those related to water exposure) altogether. As the project uses existing antibiotics and modified-release techniques for a new application, better treatment of glue ear could be developed at very little cost, and the common nature of OME means that many children could benefit within 5-10 years following this research.

11.5 EFFECTIVENESS OF ANTIBIOTIC PELLETS: CONCLUSION

The understanding that biofilms play a key role in the aetiopathogenesis of OME opens the possibility of better treatments, particularly aimed at reducing the high need for repeat VT insertion. The modified-release antibiotic pellets studied here can release antibiotics for up to 3 weeks, and can eradicate biofilms in an *in vitro* model. Although further developments are necessary, the results indicate that such a strategy could, in principle, be a useful new treatment against OME biofilms.

12 SUMMARY

OTITIS MEDIA WITH EFFUSION: CURRENT TREATMENT, NEW UNDERSTANDING OF ITS AETIOPATHOGENESIS, AND A NOVEL THERAPEUTIC APPROACH

Otitis media with effusion is the commonest cause of hearing loss in childhood, and its treatment with ventilation tubes one of the commonest indications for surgery. Despite the ubiquitous nature of OME, many questions surround its aetiology and treatment, whilst better therapies are clearly required as many children will require surgery more than once.

The clinical part of this thesis analysed children enrolled in a clinical trial of VTs versus VTs plus adenoidectomy as a treatment for OME, with a minimum follow up period of 7.5 years from randomisation. Of the children randomised to VTs, 63.6% required VTs again, and even with additional adenoidectomy the need for repeat surgery remains high. Hence, ventilation tubes have clear deficiencies. The guidelines setting out how the current treatment should be applied are also not followed, as only 32.2% of children have surgery in accordance with the criteria set out in NICE guidelines, although the proportion meeting guidelines' criteria is increased to 87.0% if children with exceptional circumstances are included.

Better treatment is therefore clearly required, but the first step towards this is a clear understanding of OME aetiopathogenesis. Much controversy has

surrounded the role of bacteria and biofilms in OME, but this thesis has shown that live bacteria can be demonstrated in 91.9% of middle ear effusions (using culture and confocal microscopy); further, biofilms were identified in 49% of those effusions that contained live bacteria on confocal microscopy. Others have also shown demonstrated biofilms in OME, and many of the clinical characteristics of OME are typical of biofilm infections.

Understanding that bacteria and biofilms are important in OME aetiology opens up potential avenues for better treatments directed against biofilms, with the aim of improving the care of children with OME, particularly the high rate of repeat surgery. To investigate high-dose antibiotics as a treatment for OME, a *Staphylococcus aureus* biofilm model was developed, and used to show that biofilm eradication requires antibiotic (rifampicin and clindamycin) levels 1,000 times higher that those required to inhibit planktonic bacteria, over a period of 2-3 weeks. Such high levels may be difficult to achieve in the middle ear, at least without systemic effects, and a local delivery strategy using biodegradable modified-release antibiotic pellets (PLGA, CMC or pluronic, rifampicin and clindamycin) was proposed. Drug release from these pellets was investigated with High Performance Liquid Chromatography and Serial Plate Transfer Testing, which demonstrated that antibiotics can be released for up to 3 weeks. Importantly, the pellets were able to eradicate biofilms in the *in vitro* model.

Hence, this thesis has shown that current OME treatment has significant disadvantages, but better understanding of OME pathogenesis raises the possibility of rational new therapeutic strategies.

Although the findings are encouraging, much further research is needed before this can be translated into patient benefit. Drug release requires further optimisation, and the use of additional antibiotics to achieve effectiveness against other bacteria is essential before a clinical trial can be planned.

To conclude, current OME treatment has significant shortcomings, but better understanding of OME aetiopathogenesis opens up the possibility of novel therapeutic strategies. Biodegradable antibiotic pellets designed to eradicate OME biofilms may be a new, better treatment that could greatly improve the care of children with OME in the future.

13 PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS WORK

PUBLICATIONS

1. National Institute for Clinical Excellence guidelines on the surgical management of Otitis Media with Effusion: are they being followed and have they changed practice? M Daniel, T Kamani, S El-Shunnar, MC Jaberoo, A Harrison, S Yalamanchili, L Harrison, WS Cho, N Fergie, R Bayston, JP Birchall. Int J Paed Otorhinolaryngol 2013; 77: 54-58
2. Biofilm eradication with biodegradable modified-release antibiotic pellets: a potential treatment for Glue Ear. M Daniel, R Chessman, S Al-Zahid, B Richards, C Rahman, W Ashraf, J M^{c}Laren, H Cox, O Qutachi, H Fortnum, N Fergie, K Shakesheff, JP Birchall, R Bayston. Arch Otolaryngol Head Neck Surg 2012; 138(10): 942-949.
3. Bacterial Infection in Otitis Media with Effusion. M Daniel, S Imtiaz-Umer, N Fergie, J Birchall, R Bayston. Int J Paed Otorhinolaryngol 2012; 76(10): 1416-1422
4. The treatment of glue ear using biodegradable polymers to deliver high dose antibiotics and mucolytics to infection site. S Al-Zahid, M Daniel, R Chessman, W Ashraf, H Fortnum, K Shakesheff, J Birchall, R Bayston. Int J Surg 2011; 9(7): 496 (abstract)
5. Development of an in-vitro model of Staphylococcus aureus biofilm infection with a focus on reduced susceptibility to antibiotics. M Daniel, C Rahman, W Ashraf, N Fergie, K Shakesheff, JP Birchall, R Bayston. J Hosp Infect 2010; 76 (Suppl 1): S4 (abstract)

6. Are We Following National Guidelines on Management of OME? M Daniel, T Kamani, S El-Shunnar, A Harrison, J Birchall. Otolaryngol Head Neck Surg 2010; 143 (2, Suppl 2): P255 (abstract)
7. Bacterial Infection in Otitis Media with Effusion. S Imtiaz-Umer, M Daniel, N Fergie, J Birchall, R Bayston. Otolaryngol Head Neck Surg 2010; 143 (2, Suppl 2): P86 (abstract)

INTERNATIONAL PRESENTATIONS

1. Have National Institute for Clinical Excellence guidelines on management of Otitis Media with Effusion changed practice? M Daniel, T Kamani, S El-Shunnar, M Jaberoo, A Harrison, S Yalamanchili, L Harrison, WS Cho, N Fergie, R Bayston, JP Birchall. ESPO, Amsterdam, Jun 12 (oral)
2. Eradication of biofilms using a biodegradable controlled-release antibiotic formulation. M Daniel, R Chessman, S Al-Zahid, B Richards, C Rahman, H Fortnum, N Fergie, K Shakesheff, R Bayston, JP Birchall. COSM / ASPO, San Diego, Apr 12 (oral). **Potsic Basic Science Prize runner up**
3. Eradication of biofilms using a biodegradable controlled-release antibiotic formulation. M Daniel, R Chessman, S Al-Zahid, B Richards, C Rahman, W Ashraf, J M^{c}Laren, H Cox, O Qutachi, H Fortnum, N Fergie, K Shakesheff, JP Birchall, R Bayston. Australian and New Zealand Society for Paediatric Otolaryngology, Brisbane, Sept 11 (oral)
4. Have National Institute for Clinical Excellence guidelines on the surgical management of Otitis Media with Effusion affected adenoidectomy rates? T Kamani, M Daniel, S El-Shunnar, M Jaberoo, A Harrison, S Yalamanchili, L

Harrison, WS Cho, N Fergie, R Bayston, JP Birchall. Confederation of European ORL-HNS, Barcelona, Jul 11 (oral)

5. National Institute for Clinical Excellence guidelines on the surgical management of Otitis Media with Effusion: are they being followed and have they changed practice? M Daniel, T Kamani, S El-Shunnar, M Jaberoo, A Harrison, S Yalamanchili, L Harrison, WS Cho, N Fergie, R Bayston, JP Birchall. Recent Advances in Otitis Media, New Orleans, June 11 (oral). **Best oral presentation runner up**.
6. Biodegradable controlled-release antibiotic middle ear implant for the treatment of Otitis Media with Effusion. R Chessman, M Daniel, C Rahman, W Ashraf, S Al-Zahid, J M^{c}Laren, B Richards, H Cox, H Fortnum, N Fergie, K Shakesheff, JP Birchall, R Bayston. Recent Advances in Otitis Media, New Orleans, June 11 (poster)
7. Treating glue ear biofilms: an *in vitro* model. M Daniel, C Rahman, W Ashraf, S Al-Zahid, J McLaren, H Cox, H Fortnum, N Fergie, K Shakesheff, JP Birchall, R Bayston. Recent Advances in Otitis Media, New Orleans, June 11 (poster)
8. Development of an *in vitro* model of *Staphylococcus aureus* biofilm infection with a focus on reduced susceptibility to antibiotics. M Daniel, C Rahman, W Ashraf, N Fergie, K Shakesheff, JP Birchall, R Bayston. International Conference of the Hospital Infection Society, Liverpool, Oct 10 (oral)
9. Are we following national guidelines on management of OME? M Daniel, T Kamani, S El Shunnar, A Harrison, JP Birchall. American Academy, Boston, Sept 10 (poster)

10. Bacterial infection in Otitis Media with Effusion. S Imtiaz-Umer, M Daniel, R Bayston, N Fergie, J Birchall. American Academy, Boston, Sept 10 (oral)
11. New challenges and the role of biofilms. JP Birchall, R Bayston, S Imtiaz-Umer, M Daniel, N Fergie. 3rd International Congress of Otology-Rhinology & Skull Base Surgery Current Concepts, Athens, May 09 (oral)
12. Does the benefit of +adenoidectomy with ventilation tube insertion persist long-term? M Daniel, H Vaghela, C Philpott, RSA Thomas, M Gannon, H Spencer, M Haggard. 9th International Symposium on Recent Advances in Otitis Media. Florida, USA, Jun 07 (oral)

NATIONAL PRESENTATIONS

1. Eradication of glue ear biofilms using a biodegradable controlled-release antibiotic formulation. M Daniel, R Chessman, S Al-Zahid, B Richards, C Rahman, W Ashraf, J M^{c}Laren, H Cox, O Qutachi, H Fortnum, N Fergie, K Shakesheff, JP Birchall, R Bayston. **RSM trainee of the year finalists'** presentations, Nov 11
2. Treatment of glue ear biofilms with antibiotics and mucolytics. S Al-Zahid, M Daniel, R Chessman, W Ashraf, N Fergie, K Shakesheff, H Fortnum, JP Birchall, R Bayston. Association of Surgeons in Training, April 11 (Oral)
3. The *in-vitro* treatment of glue ear biofilms using N-acetylcysteine in combination with rifampicin and clindamycin. Al-Zahid S, Daniel M, Rahman C, Ashraf W, Richards B, Fergie N, Chessman R, Fortnum H, Shakesheff K, Bayston R, Birchall JP. BSAC, March 11 (poster)
4. Biodegradable controlled-release antibiotic middle ear implant for the treatment of Otitis Media with Effusion. R Chessman, M Daniel, C

Rahman, W Ashraf, S Al-Zahid, J M^{c}Laren, B Richards, H Cox, H Fortnum, N Fergie, K Shakesheff, JP Birchall, R Bayston. ORS, March 11 (oral). **Junior presenters prize won by medical student co-supervised by me**

5. Are we following National Institute for Clinical Excellence guidelines on the surgical management of Otitis Media with Effusion? A comparison of five different centres. WS Cho, M Daniel, L Harrison, T Kamani, S El-Shunnar, M Jaberoo, A Harrison, S Yalamanchili, N Fergie, R Bayston, JP Birchall. ORS, March 11 (oral)
6. Eradication of glue ear biofilms using a biodegradable controlled-release antibiotic formulation. M Daniel, R Chessman, S Al-Zahid, B Richards, C Rahman, W Ashraf, J M^{c}Laren, H Cox, O Qutachi, H Fortnum, N Fergie, K Shakesheff, JP Birchall, R Bayston. RSM Otology, March 11. **RSM ENTEx short papers prize.**
7. Treating Glue Ear Biofilms: an *in vitro* model. M Daniel, C Rahman, W Ashraf, N Fergie, K Shakesheff, JP Birchall, R Bayston. Midland Institute of Otorhinolaryngology, Birmingham, Jan 11 (oral presentation of funded research)
8. Have NICE guidelines on the surgical management of Otitis Media with Effusion changed clinical practice? A multicentre before and after comparison. M Daniel, T Kamani, S El-Shunnar, M Jaberoo, A Harrison, S Yalamanchili, L Harrison, WS Cho, N Fergie, R Bayston, JP Birchall. BAPO, London, Sept 10 (oral). **Susanna Leighton Prize for best essay submission to BAPO**

9. Treating Glue Ear Biofilms: an *in vitro* model. M Daniel, C Rahman, W Ashraf, N Fergie, K Shakesheff, JP Birchall, R Bayston. ORS / ENTUK, Coventry, Sept 10 (oral). **Angell-James prize for best free paper**
10. Treating Glue Ear Biofilms: an *in vitro* model. M Daniel, C Rahman, W Ashraf, N Fergie, K Shakesheff, JP Birchall, R Bayston. Society for General Microbiology, Nottingham, Sept 10 (poster)
11. Bacterial infection in Otitis Media with Effusion: Confocal microscopy combined with microbiological culture demonstrates live bacteria in >90% of middle ear effusion samples. M Daniel, S Imtiaz-Umer, R Bayston, N Fergie, J Birchall. ORS, Oxford, Mar 10 (oral)
12. Are we complying with NICE guidance on the surgical management of OME? WS Cho, M Daniel, JP Birchall. ENT UK, London, Sept 09 (poster)
13. Does the benefit of adenoidectomy in addition to ventilation tube insertion persist long-term? M Daniel, H Vaghela, C Philpott, RSA Thomas, M Gannon, H Spencer, M Haggard. ORS, London, Sept 06 (oral)
14. Health care needs of children with established glue ear. M Daniel, H Vaghela, C Philpott, RSA Thomas, M Gannon, H Spencer, M Haggard. BAPO, Birmingham, Sept 06 (oral)

14 REFERENCES

Adams, SB, MF Shamji, DL Nettles, P Hwang, and LA Setton. "Sustained Release of Antibiotics from Injectable and Thermally Responsive Polypeptide Depots." *J Biomed Mater Res B Appl Biomater 90(1): 67–74*, 2009.

Aldridge, KE, and CW Stratton. "Bactericidal activity of ceftizoxime, cefotetan, and clindamycin against cefoxitin-resistant strains of the Bacteroides fragilis group." *J Antimicrob Chemother 28(5): 701-705*, 1991.

Al-Hussaini, A, D Owens, and A Tomkinson. "Have two UK national guidelines had any effect on grommet day-case utilisation and rate over the last 10 years?" *Eur Arch Otorhinolaryngol, in press*, 2011.

Almaguer-Flores, A, LA Ximénez-Fyvie, and SE Rodil. "Oral bacterial adhesion on amorphous carbon and titanium films: effect of surface roughness and culture media." *J Biomed Mater Res B Appl Biomater 92(1): 196-204*, 2010.

Al-Zahid, S, et al. "The treatment of glue ear using biodegradable polymers to deliver high dose antibiotics and mucolytics to infection site." *Int J Surg 9(7): 496*, 2011.

Anderson, EM, et al. "Sustained release of antibiotic from poly(2-hydroxyethyl methacrylate) to prevent blinding infections after cataract surgery." *Biomaterials 30: 5675-5681*, 2009.

Anderson, JM, and SW Kim. *Recent advances in drug delivery systems.* New York: Plenum press, 1984.

Antonelli, PJ, EM Sampson, and DM Lang. "Safety and efficacy of carbomethylcellulose foam in guinea pig middle ear surgery." *Otolaryngol Head Neck Surg 142(3): 405-408*, 2010.

Aqualon. "Sodium carboxymethylcellulose chemistry." *http://www.pshcanada.com/Tech%20Data/Gum%20CMC%20-%20chem.htm, accessed 30th May 2011*, 2000.

Arthrocare. "CMC Technology." *http://www.arthrocareent.com/cmc_technology, accessed 28th May 2011*, 2011.

Aslam, S. "Effect of antibacterials on biofilms." *Am J Infect Control 36: S175.e9-S175.e11*, 2008.

Aslam, S, BW Trautner, V Ramanathan, and RO Darouiche. "Combination of tigecycline and N-acetylcysteine reduces biofilm-embedded bacteria on vascular catheters." *Antimicrob Agents Chemother 51(4): 1556-1558*, 2007.

Bajpai, A, S Shukla, R Saini, and A Tiwari. *Stimuli responsive drug delivery systems: from introduction to application.* Shrewsbury: Smithers, 2010.

Bakker-Woudenberg, IA, W van Vianen, D van Soolingen, HA Verbrugh, and MA van Agtmael. "Antimycobacterial agents differ with respect to their bacteriostatic versus bactericidal activities in relation to time of exposure, mycobacterial growth phase, and their use in combination." *Antimicrob Agents Chemother 49(6): 2387-2398*, 2005.

Bak-Pedersen, K, and M Tos. "Distribution and density of goblet cells in the middle ear in children." *Anat Anz 139(5): 455-467*, 1976.

Barakate, M, E Beckenham, J Curotta, and M da Cruz. "Bacterial biofilm adherence to middle-ear ventilation tubes: scanning electron micrograph images and literature review." *J Laryngol Otol 121(10): 993-997*, 2007.

Barbucci, R, G Leone, and A Vecchiullo. "Novel carboxymethylcellulose-based microporous hydrogels suitable for drug delivery." *J Biomater Sci Polym Ed 15(5): 607-619*, 2004.

Barriere, SL. "Protein synthesis inhibitors, fluoroquinolones and rifampin for biofilm infections." In *Biofilms, infection, and antimicrobial therapy*, edited by Rupp ME, Finch RG Pace JL. Boca Raton: Taylor&Francis, 2006.

Barringer. "Coefficient of variation." *http://www.barringer1.com/feb98prb.htm, accessed 21st may 2011*, 1998.

Bassler, BL, and R Losick. "Bacterially speaking." *Cell 125: 237-246*, 2006.

Bayston, R, and J Rodgers. "Production of extra-cellular slime by Staphylococcus epidermidis during stationary phase of growth: its association with adherence to implantable devices." *J Clin Pathol 43(10): 866-870*, 1990.

Bayston, R, et al. "Biofilm formation by Propionibacterium acnes on biomaterials in vitro and in vivo: impact on diagnosis and treatment." *J Biomed Mater Res 81A: 705–709*, 2007.

Bayston, R, L Vera, and W Ashraf. "Activity of an Antimicrobial Hydrocephalus Shunt Catheter against Propionibacterium acnes." *Antimicrob Agents Chemother 54(12): 5082-5085*, 2010.

Bayston, R, LE Fisher, and K Weber. "An antimicrobial modified silicone peritoneal catheter with activity against both Gram-positive and Gram-negative bacteria." *Biomaterials 30(18): 3167-3173*, 2009.

Bayston, R, N Grove, J Siegel, D Lawellin, and S Barsham. "Prevention of hydrocephalus shunt catheter colonisation in vitro by impregnation with antimicrobials." *J Neurol Neurosurg Psychiatry 52(5): 605-609*, 1989.

Bayston, R, W Ashraf, and C Bhundia. "Mode of action of an antimicrobial biomaterial for use in hydrocephalus shunts." *J Antimicrob Chemother 53(5): 778-782*, 2004.

Becher, P. *Emulsions: Theory and practice.* Oxford: Oxford University Press, 2001.

Beckman-Coulter. "http://www.beckmancoulter.com/coultercounter/showDoc/doc.do?filename=&fileid=10077." *accessed 24th Dec 2010*, 2010.

Berney, M, F Hammes, F Bosshard, HU Weilenmann, and T Egli. "Assessment and Interpretation of Bacterial Viability by Using the LIVE/DEAD BacLight Kit in Combination with Flow Cytometry." *Appl Environ Microbiol 73(10): 3283–3290*, 2007.

Bernthal, NM, et al. "A mouse model of post-arthroplasty Staphylococcus aureus joint infection to evaluate in vivo the efficacy of antimicrobial implant coatings." *PLoS One 5(9): e12580*, 2010.

Bhutani, H, TT Mariappan, and S Singh. "The physical and chemical stability of anti-tuberculosis fixed-dose combination products under accelerated climatic conditions." *Int J Tuberc Lung Dis 8(9): 1073-1080*, 2004.

BiosurfaceTechnologies. "Biofilm reactors." *http://www.biofilms.biz/biofilm-reactors, accessed 20th Sept 2011*, 2011.

Black, N. "Geographical variations in use of surgery for glue ear." *J Roy Soc Med 78: 641-648*, 1985.

Black, N, and A Hutchings. "Reduction in the use of surgery for glue ear: did national guidelines have an impact?" *Qual Saf Health Care 11: 121–124*, 2002.

Black, NA, CFB Sanderson, AP Freeland, and MP Vessey. "A randomised controlled trial of surgery for glue ear." *Br Med J 300: 1551-1556*, 1990.

Bluestone, CD, JS Stephenson, and LM Martin. "Ten-year review of otitis media pathogens." *Pediatr Infect Dis J 11(8 Suppl): S7-11*, 1992.

Bober, C. "Quantification of single-species marine biofilm with Alcian Blue." *J Young Investigator 12(4). retrieved 12 March 2011 from http://www.jyi.org/research/re.php?id=149*, 2005.

Boles, BR, and AR Horswill. "Staphylococcal biofilm disassembly." *Trends Microbiol 19(9): 449-455*, 2011.

Bonding, P, and M Tos. "Grommets versus paracentesis in secretory otitis media. A prospective, controlled study." *Am J Otol 6(6): 455-460*, 1985.

Bondy, J, S Berman, J Glazner, and D Lezotte. "Direct expenditures related to otitis media diagnosis: extrapolations from a pediatric Medicaid cohort." *Pediatrics 105: e72*, 2000.

Bonhoeffer, S, M Lipsitch, and BR Levin. "Evaluating treatment protocols to prevent antibiotic resistance." *Proc Natl Acad Sci U S A 94(22): 12106-12111*, 1997.

Bonkat, G, et al. "Improved detection of microbial ureteral stent colonisation by sonication." *World J Urol 29(1):v133-138*, 2010.

Bossy, L, S Erickson, R Gurny, and F Delie. "Intraocular implants for controlled drug delivery." In *Modified-release drug delivery technology*, edited by Rathbone MJ. New York: Informa, 2008.

Brook, I. "Veillonella infections in children." *J Clin Microbiol*, 1996: 34: 1283–1285.

Brown, MJ, SH Richards, and AG Ambegaokar. "Grommets and glue ear: a five-year follow up of a controlled trial." *J R Soc Med 71(5): 353-356*, 1978.

Browning, GG. "Adjuvant adenoidectomy in persistent bilateral otitis media with effusion: hearing and revision surgery outcomes through 2 years in the TARGET randomised trial." *Clin Otolaryngol, in press*, 2012.

BSAC British Society for Antimicrobial Chemotherapy. "www.bsac.org.uk, accessed 9th July 2010." 2010.

Bunse, T, H Hildmann, W Zan, and W Opferkuch. "A bacteriological study of otitis media with effusion. Concurrent coagulase-negative staphylococcal infections in the middle ear." *Arch Otorhinolaryngol*, 1987: 243(6): 387-91.

Cammarota, G, et al. "Biofilm demolition and antibiotic treatment to eradicate resistant Helicobacter pylori: a clinical trial." *Clin Gastroenterol Hepatol 8(9): 817-820.e3*, 2010.

Campoccia, D, L Montanaro, P Speziale, and CR Arciola. "Antibiotic-loaded biomaterials and the risks for the spread of antibiotic resistance following their prophylactic and therapeutic use." *Biomaterials 31: 6366-6377*, 2010.

Canales, BK, L Higgins, T Markowski, L Anderson, QA Li, and M Monga. "Presence of five conditioning film proteins are highly associated with early stent encrustation." *J Endourol 23: 1437-1442*, 2009.

Cao, Z, and Y Sun. "Chitosan-based rechargable long-term antimicrobial and biofilm controlling systems." *J Biomed Mater Res A 89: 960-967*, 2009.

Caro, A, et al. "Bioengineering of stainless steel surface by covalent immobilization of enzymes. Physical characterization and interfacial enzymatic activity." *J Colloid Interface Sci 349(1): 13-18*, 2010.

Casselbrant, ML, EM Mandel, HE Rockette, M Kurs-Lasky, PA Fall, and CD Bluestone. "Adenoidectomy for otitis media with effusion in 2-3-year-old children." *Int J Pediatr Otorhinolaryngol 73: 1718-1724*, 2009.

Catena, E, G Perez, B Sadaba, JR Azanza, and Campanero MA. "A fast reverse-phase high performance liquid chromatographic tandem mass spectrometry assay for the quantification of clindamycin in plasma and saliva using a rapid resolution package." *J Pharmaceut Biomed Analysis 50: 649–654*, 2009.

Ceri, H, ME Olson, C Stremick, RR Read, D Morck, and A Buret. "The Calgary Biofilm Device: New technology for rapid determination of antibiotic susceptibilities of bacterial biofilms." *J Clin Microbiol 37(6): 1771–1776*, 1999.

Chae, JM, SM Mo, and IJ Oh. "*Effects of poloxamer 188 on* the characteristics of poly(lactide-co-glycolide) nanoparticles." *J Nanosci Nanotechnol 10(5): 3224-3227*, 2010.

Chaignon, P, I Sadovskaya, CH Ragunah, N Ramasubbu, JB Kaplan, and S Jabbouri. "Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition." *Appl Microbiol Biotechnol 75(1): 125-132*, 2007.

Chen, K, X Wu, G Jiang, J Du, and H Jiang. "Low dose macrolide administration for long term is effective for otitis media with effusion in children." *Auris Nasus Larynx in press*, 2012.

Cheow, WS, MW Chang, and K Hadinoto. "Antibacterial efficacy of inhalable levofloxacin-loaded polymeric nanoparticles against E. coli biofilm cells: The Effect of antibiotic release profile." *Pharm Res in press*, 2010.

Child and Maternal Health Observatory. "Rates of grommet surgery aged 17 and under." *http://atlas.chimat.org.uk, accessed 3rd May 2012*, 2012.

Coates, H, R Thornton, J Langlands, P Filion, AD Keil, S Vijayasekaran,P Richmond. "The role of chronic infection in children with otitis media with effusion: evidence for intracellularpersistence of bacteria." *Otolaryngol Head Neck Surg 138(6):778-81, 2008.*

Cole, SP, J Harwood, R Lee, R She, and DG Guiney. "Characterization of monospecies biofilm formation by Helicobacter pylori." *J Bacteriol 186(10): 3124-3132*, 2004.

Coyte, P, R Croxford, C Asche, T To, W Feldman, and J Friedberg. "Physician and population determinants of rates of middle-ear surgery in Ontario." *JAMA 286(17): 2128-2135*, 2001.

Cu, Y, and WM Saltzman. "Controlled surface modification with poly(ethylene)glycol enhances diffusion of PLGA nanoparticles in human cervical mucus." *Mol Pharm 6(1): 173–181*, 2009.

Czekaj, J, et al. "Efficacy of combined oral clindamycin/rifampicin regimen for therapy of staphylococcal osteoarticular infections." *Scand J Infect Dis 43(11-12): 962-967*, 2011.

D'Agata, EM, M Dupont-Rouzeyrol, P Magal, D Olivier, and S Ruan. "The impact of different antibiotic regimens on the emergence of antimicrobial-resistant bacteria." *PLoS One 3(12): e4036*, 2008.

Dagostino, L, AE Goodman, and KC Marshall. "Physiological responses induced in bacteria adhering to surfaces." *Biofouling 4(1): 113-119*, 1994.

Daly, KA, LL Hunter, BR Lindgren, R Margolis, and GS Giebink. "Chronic otitis media with effusion sequelae in children treated with tubes." *Arch Otolaryngol Head Neck Surg 129(5): 517-522*, 2003.

Darouiche, RO. "Device-associated infections: a macroproblem that starts with microadherence." *Clin Infect Dis 33(9): 1567-1572*, 2001.

Datta, D. "http://nrsmedic.blogspot.com.au/ accessed 10th May 2012." 2012.

del Prado, G, V Ruiz, P Naves, V Rodríguez-Cerrato, F Soriano, and M del Carmen Ponte. "Biofilm formation by Streptococcus pneumoniae strains and effects of human serum albumin, ibuprofen, N-acetyl-l-cysteine, amoxicillin, erythromycin, and levofloxacin." *Diagn Microbiol Infect Dis 67(4): 311-318*, 2011.

Dempster, JH, GG Browning, and SG Gatehouse. "A randomized study of the surgical management of children with persistent otitis media with effusion associated with a hearing impairment." *J Laryngol Otol 107(4): 284-289*, 1993.

Desai, SD, and J Blanchard. "In Vitro Evaluation of Pluronic F127-Based Controlled-Release Ocular Delivery Systems for Pilocarpine." *J Pharmaceut Sci 87(2): 226-230*, 1998.

Diggle, SP, SA Crusz, and M Camara. "Quorum sensing." *Curr Biol 17(21): R907-910*, 2007.

Djordjevic, D, M Wiedmann, and LA McLandsborough. "Microtiter plate assay for assessment of Listeria monocytogenes biofilm formation." *Applied Environ Microbiol 68 (6): 2950–2958*, 2002.

Donlan, R. "Biofilm formation: a clinically relevant microbiological process." *Clinical Infectious Diseases 33:1387–1392*, 2001.

Donlan, RM, and JW Costerton. "Biofilms: survival mechanisms of clinically relevant microorganisms." *Clin Microbiol Rev 15(2): 167-193, 2002.*

Dorati, R, et al. "Gamma-Irradiation of PEGd,lPLA and PEG-PLGA multiblock copolymers. I. Effect of irradiation doses." *AAPS PharmSciTech 9(2): 718-725*, 2008.

Doyle, WJ, and JD Swarts. "Eustachian tube-Tensor veli palatini muscle-cranial base relationships in children and adults: an osteological study." *Int J Pediatr Otorhinolaryngol 74(9): 986-990*, 2010.

Dumond, P, P Franck, M Morisset, J Sainte Laudy, G Kanny, and DA Moneret-Vautrin. "Pre-lethal anaphylaxis to carboxymethylcellulose confirmed by identification of specific IgE--review of the literature." *Eur Ann Allergy Clin Immunol 41(6): 171-176*, 2009.

Dumortier, G, JL Grossiord, F Agnely, and JC Chaumeil. "A review of poloxamer 407 pharmaceutical and pharmacological characteristics." *Pharm Res 23(12): 2709-2728*, 2006.

Ecker, DE, JJ Drader, and J Gutierrez. "The Ibis T5000 universal biosensor: an automated platform for pathogen identification and strain typing." *J Assoc Lab Automation 11: 341–345*, 2006.

Emeje, MO, OO Kunle, and SI Ofoefule. "Effect of the molecular size of carboxymethylcellulose and some polymers on the sustained release of theophylline from a hydrophilic matrix." *Acta Pharm 56: 325–335*, 2006.

Ertan, G, O Ozer, E Baloğlu, and Güneri. "Sustained-release microcapsules of nitrofurantoin and amoxicillin; preparation, in-vitro release rate, kinetic and micromeritic studies." *J Microencapsul 14(3): 379-388*, 1997.

Escobar-Chávez, JJ, M López-Cervantes, A Naïk, YN Kalia, D Quintanar-Guerrero, and A Ganem-Quintanar. "Applications of thermo-reversible pluronic F-127 gels in pharmaceutical formulations." *J Pharm Pharm Sci 9(3): 339-358*, 2006.

Faisanta, N, J Akikia, F Siepmannb, JP Benoita, and J Siepmann. "Effects of the type of release medium on drug release from PLGA-based microparticles: Experiment and theory." *Int J Pharmaceutics 314(2): 189-197*, 2006.

Feng, H, J Sun, and P Jiang. "In vitro and in vivo biodegradation of sustained-*release vehicle poloxamer* 407 in situ gel." *Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi 22(1): 28-31*, 2008.

Feng, HY, JJ Sun, W Jiang, and P Jiang. "Effect of poloxamer 407 on the cochlear morphology and hearing function after perfusion in round window: experiment with guinea pigs." *Zhonghua Yi Xue Za Zhi 87(32): 2289-2291*, 2007.

Fergie, N, R Bayston, JP Pearson, and JP Birchall. "Is otitis media with effusion a biofilm infection?" *Clin Otolaryngol 29(1): 38-46*, 2004.

Flint, PW, et al. *Cummings Otolaryngology: Head and Neck Surgery.* Philadelphia: Elsevier Mosby, 2010.

Foreman, A, J Jervis-Bardy, and PJ Wormwald. "Do biofilms contribute to the initiation and recalcitrance of chronic sinusitis?" *Laryngoscope 121: 1085-1091*, 2011.

Freemantle, N, EL Harvey, F Wolf, JM Grimshaw, R Grilli, and LA Bero. "Printed educational materials: effects on professional practice and health care outcomes." *Cochrane Database Syst Rev*, 2000.

Friess, W, and M Schlapp. "Sterilization of gentamicin containing collagen/PLGA microparticle composites." *Eur J Pharm Biopharm 63(2): 176-187*, 2006.

Furno, F, et al. "Silver nanoparticles and polymeric medical devices: a new approach to prevention of infection?" *J Antimicrob Chemother 54(6): 1019-1024*, 2004.

Galli, J, et al. "Damage to ciliated epithelium in chronic rhinosinusitis: what is the role of bacterial biofilms?" *Ann Otol Rhinol Laryngol 117(12): 902-908*, 2008.

Garty, S, et al. "Sustained antibiotic release from an intraocular lens-hydrogel assembly for cataract surgery." *Invest Ophthal Vis Sci 52: 6109-6116*, 2011.

Gates, GA, CA Avery, and TJ Prihoda. "Effectiveness of adenoidectomy and tympanostomy tubes in the treatment of chronic otitis media with effusion." *NEJM 1444-1451*, 1987.

Gavini, E, L Manunta, S Giua, G Achenza, and P Giunchedi. "Spray-Dried Poly(D,L-Lactide) Microspheres Containing Carboplatin for Veterinary Use: In Vitro and In Vivo Studies." *AAPS PharmSciTech 6(1): Article 17*, 2005.

Gok, U, Y Bulut, E Keles, S Yalcin, and MZ Doymaz. "Bacteriological and PCR analysis of clinical material aspirated from otitis media with effusions." *Int J Pediatr Otorhinolaryngol 60(1):49-54*, 2001.

Gomes, F, P Teixeira, H Ceri, and R Olivieira. "Evaluation of antimicrobial activity of certain combinations of antibiotics against in vitro Staphylococcis epidermidis biofilms." *Indian J Med Res 135(4): 542-547*, 2012.

Goycoolea, M, DC Muchow, LM Sirvio, and RM Winandy. "In search of missing links in otology. II. development of an implantable middle ear drug delivery system: initial studies of sustained ampicillin release for the treatment of otitis media." *Laryngoscope 101(7 pt1): 727-732*, 1991.

Goycoolea, M, DC Muchow, LM Sirvio, RM Winandy, DM Canfax, and M Hueb. "Extended middle ear drug delivery. A new concept; a new device." *Acta Otolaryngol Suppl 493: 119-126*, 1992.

Goycoolea, MV, and DC Muchow. "Sustained release of antimicrobials in the middle ear using a biodegradable support." *Ann Otol Rhinol Laryngol Suppl 163: 46-48*, 1994.

Haapkyla, J, G Karevold, KJ Kvaerner, and A Pitkaranta. "Trends in otitis media surgery: a decrease in adenoidectomy." *Int J Pediatr Otolaryngol 72(8) 1270-1213*, 2008.

Hall, AJ, AR Maw, and CD Steer. "Developmental outcomes in early compared with delayed surgery for glue ear up to age 7 years: a randomised controlled trial." *Clin Otolaryngol* 34 (2009): 12-20.

Hall-Stoodley, L, and P Stoodley. "Evolving concepts in biofilm infections." *Cellular Microbiology 11(7): 1034–1043*, 2009.

Hall-Stoodley, L, et al. "Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media." *JAMA 296(2): 202-211*, 2006.

Hardisty-Hughes, RE, et al. "A mutation in the F-box gene, Fbxo11, causes otitis media in the Jeff mouse." *Hum Mol Genet 15(22): 3273-3279*, 2006.

He, Z, et al. "Detection of gastric pepsin in middle ear fluid of children with otitis media." *Otolaryngol Head Neck Surg 137(1): 59-64*, 2007.

Hekiert, AM, et al. "Biofilms correlate with TH1 inflammation in the sinonasal tissue of patients with chronic rhinosinusitis." *Otolaryngol Head Neck Surg 141(4): 448-453*, 2009.

Helfaer, MA, et al. "Polysomnography after adenostonsillectomy in mild pediatric obstructive sleep apnea." *Crit Care Med 24: 1323-1327*, 1996.

Hendolin, PH, A Markkanen, J Ylikoski, and JJ Wahlfors. "Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions." *J Clin Microbiol 35: 2854-2858*, 1997.

Herrmann, J, and R Bodmeier. "Biodegradable, somatostatin acetate containing microspheres prepared by various aqueous and non-aqueous solvent evaporation methods." *Eur J Pharmaceu Biopharmaceu 45: 75–82*, 1998.

HES Hospital Episode Statistics. 2010: www.hesonline.nhs.uk.

Hiremath, PS, and RN Saha. "Oral Controlled Release Formulations of Rifampicin. Part II: Effect of Formulation Variables and Process Parameters on In Vitro Release." *Drug Delivery 15: 159–168*, 2008.

Hoefler, AC. "Sodium carboxymethylcellulose: chemistry, functionality and applications." *http://class.fst.ohio-state.edu/fst621/Additive%20classes/cmctlk.pdf, accessed 30th May 2011*, 2011.

Homøe, P, T Bjarnsholt, M Wessman, HC Sørensen, and HK Johansen. "Morphological evidence of biofilm formation in Greenlanders with chronic

suppurative otitis media." *Eur Arch Otorhinolaryngol 266(10): 1533-1538*, 2009.

Horie, RT, et al. "Sustained delivery of lidocaine into the cochlea using poly lactic/glycolic acid microparticles." *Laryngoscope 120(2): 377-383*, 2010.

Hyden, D, B Akerlind, and M Peebo. "Inner ear and facial nerve complications of acute otitis media with focus on bacteriology and virology." *Acta Otolaryngol*, 2006: 126(5): 460-6.

Igartua, M, RM Hernández, JE Rosas, ME Patarroyo, and JL Pedraz. "Gamma-irradiation effects on biopharmaceutical properties of PLGA microspheres loaded with SPf66 synthetic vaccine." *Eur J Pharm Biopharm 69(2): 519-526*, 2008.

Ikarashi, F, S Takahashi, and Y Yamamoto. "Carbon dioxide exchange via the mucosa in healthy middle ear." *Arch Otolaryngol Head Neck Surg 125(9): 975-978*, 1999.

Inchem. "http://www.inchem.org/documents/pims/pharm/rifam.htm, accessed 3rd Jan 2011." 2011.

Innovotech MBEC. *http://www.innovotech.ca/products_use.php, accessed 13/3/11*, 2011.

Invitrogen. "http://probes.invitrogen.com/media/pis/mp10318.pdf; accessed 18th March 2011." 2011.

Ishino, R, H Yoshino, Y Hirakawa, and K Noda. "Design and preparation of pulsatile release tablet as a new oral drug delivery system." *Chem Pharm Bull (Tokyo) 40(11): 3036-3041*, 1992.

Jain, D, E Carvalho, and R Banerjee. "Biodegradable hybrid polymeric membranes for ocular drug delivery." *Acta Biomater 6(4): 1370-1379*, 2010.

Jain, S, et al. "A two-stage strategy for sterilization of poly(lactide-co-glycolide) particles by γ-irradiation does not impair their potency for vaccine delivery." *J Pharm Sci 100(2): 646-654*, 2011.

Jang, CH, H Park, YB Cho, and CH Choi. "The effect of anti-adhesive packing agents on the middle ear of guinea pig." *Int J Pediatr Otorhinolaryngol 72(11): 1603-1608*, 2008.

Kadhim, AL, K Spilsbury, JB Semmens, HL Coates, and FJ Lannigan. "Adenoidectomy for Middle Ear Effusion: A Study of 50,000 Children Over 24 Years." *Laryngoscope 117: 427–433*, 2007.

Kaplan, JB. "Antibiotic-induced biofilm formation." *Int J Artif Organs 34(9): 737-751*, 2011.

Kaplan, JB, et al. "Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in staphylococci." *J Antibiot (Tokyo) 65(2): 73-77*, 2012.

Karevold, G, J Haapkyla, A Pitka, R Nafstad, and KJ Kvaerner. "Paediatric otitis media surgery in Norway." *Acta Oto-Laryngologica 127: 29-33*, 2007.

Keyhani, S, LC Kleinman, M Rothschild, JM Bernstein, R Anderson, and M Chassin. "Overuse of tympanostomy tubes in New York metropolitan area: evidence from five hospital cohort." *Br Med J 337: a1607*, 2008.

Klokkenburg, JJ, HL Hoeve, J Francke, MH Wieringa, J Borgstein, and L Feenstra. "Bile acids identified in middle ear effusions of children with otitis media with effusion." *Laryngoscope 119(2): 396-400*, 2009.

Kobayashi, H, M Oethinger, MJ Tuohy, GS Hall, and TW Bauer. "Improving Clinical Significance of PCR: Use of Propidium Monoazide to Distinguish

Viable from Dead Staphylococcus aureus and Staphylococcus epidermidis." *J Orthop Res* 27 (2009): 1243–1247.

Koga, H. "High-Performance Liquid Chromatography Measurement of Antimicrobial Concentrations in Polymorphonuclear Leukocytes." *Antimicrobial Agents Chemoth 31: 1904-1908*, 1987.

Kohler, T, GG Perron, A Buckling, and C van Delden. "Quorum Sensing Inhibition Selects for Virulence and Cooperation in Pseudomonas aeruginosa." *PLoS Pathogens 6 (5): e1000883*, 2010.

Kovaleva, J, JE Degener, and HC van der Mei. "Mimicking disinfection and drying of biofilms in contaminated endoscopes." *J Hosp Infect 76(4): 345-350*, 2010.

Kowalski, W. *Ultraviolet Germicidal Irradiation Handbook.* Berlin: Springer-Verlag, 2009.

Kubba, H, JP Pearson, and JP Birchall. "The aetiology of otitis media with effusion: a review." *Clin Otolaryngol Allied Sci*, 2000: 25(3): 181-94.

La Follette, G, J Gambertoglio, JA White, DW Knuth, and ET Lin. "Determination of clindamycin in plasma or serum by high-performance liquid chromatography with ultraviolet detection." *J Chromatogr 431: 379-388*, 1988.

Labib, ME, CJ Brumlik, P Stoodley, SS Dukhin, T Davidson, and Y Tabani. "The long-term release of antibiotics from monolithic nonporous polymer implants for use as tympanostomy tubes." *Colloids Surf A Physicochem Eng Asp 254: 331-337*, 2009.

Lane, ME, FW Okumu, and P Balausubramanian. "Injections and implants." In *Modified-release drug delivery technology*, edited by Rathbone MJ. New York: Informa, 2008.

Le, SH, J Le, Baekb, WY, and JO Lim. "Regional delivery of vancomycin using pluronic F-127 to inhibit methicillin resistant Staphylococcus aureus (MRSA) growth in chronic otitis media in vitro and in vivo." *J Controlled Release 96: 1–7*, 2004.

Lee, JS, GS, Khang, G Chae, MS Kim, SH Cho, and HB Lee. "The effect of gamma irradiation on PLGA and release behaviour of BCNU from PLGA wafer." *Macromolecular Res 11(5): 352-356*, 2003.

Lee, J-Y, KS Ko, KR Peck, WS Oh, and J-H Song. "In vitro evaluation of the antibiotic lock technique (ALT) for the treatment of catheter-related infections caused by staphylococci." *J Antimicrob Chemoth 57: 1110–1115*, 2006.

Leung, L, C Chan, J Song, B Tam, and H Naguib. "A Parametric Study on the Processing and Physical Characterization of PLGA 50/50 Bioscaffolds." *Journal of Cellular Plastics 44: 189-202*, 2008.

Lewis, K. "Riddle of Biofilm Resistance." *Antimicrob Agent Chemoth 45(4): 999–1007*, 2001.

Li, X, and B Jasti. *Design of controlled release drug delivery systems.* New York: McGraw-Hill, 2006.

Li, XJ, LY Yue, Guan XF, and SY Qiao. "The adhesion of putative probiotic lactobacilli to cultured epithelial cells and porcine intestinal mucus." *J Appl Microbiol 104(4): 1082-1091*, 2008.

Lieser, JD, and CS Derkay. "Pediatric sinusitis: when do we operate?" *Curr Opin Otolaryngol Head Neck Surg 13: 60–66*, 2005.

Lim, SJ, et al. "Effect of sodium carboxymethylcellulose and fucidic acid on the gel characterization of polyvinylalcohol-based wound dressing." *Arch Pharm Res 33(7): 1073-1081*, 2010.

Liu, KS, et al. "Steady Antibiotic Release from Biodegradable Beads in the Pleural Cavity: An In Vitro and In Vivo Study." *Chest, in press*, 2011.

Loo, SC, CP OOi, and YC Boey. "Influence of electron-beam radiation on the hydrolytic degradation behaviour of pily(lactide-co-glycolide)(PLGA)." *Biomater 26: 3809-3817*, 2005.

Lous, J, MJ Burton, J Felding, T Ovesen, M Rovers, and I Williamson. "Grommets (ventilation tubes) for hearing loss associated with otitis media with effusion in children." *Cochrane Database of Systematic Reviews*, 2005.

Lu, JM, et al. "Current advances in research and clinical applications of PLGA-based nanotechnology." *Expert Rev Mol Diagn 9(4): 325-341*, 2009.

Lyte, M, et al. "Stimulation of Staphylococcus epidermidis growth and biofilm formation by catecholamine inotropes." *Lancet*, 2003: 361(9352): 130-5.

Macfarlane, S, and JF Dillon. "Microbial biofilms in the human gastrointestinal tract." *J Appl Microbiol 102(5): 1187-1196*, 2007.

Manjunath, K, V Venkateswarlu, and A Hussain. "Preparation and characterization of nitrendipine solid lipid nanoparticles." *Pharmazie 66(3): 178-186*, 2011.

Matar, GM, N Sidani, M Fayad, and U Hadi. "Two-step PCR-based assay for identification of bacterial etiology of otitis media with effusion in infected Lebanese children." *J Clin Microbiol 36(5): 1185-1188*, 1998.

Maw, AR, and R Bawden. "The long term outcome of secretory otitis media in children and the effects of surgical treatment: a ten year study." *Acta ORL Belg 48: 317-324*, 1994.

Maw, R, and R Bawden. "Spontaneous resolution of severe chronic glue ear in children and the effect of adenoidectomy, tonsillectomy, and insertion of ventilation tubes (grommets)." *Br Med J 306: 756-760*, 1993.

McCann, MT, BF Gilmore, and SP Gorman. "Staphylococcus epidermidis device-related infections: pathogenesis and clinical management." *J Pharm Pharmacol*, 2008: 60(12): 1551-71.

McLaughlin, SC, TC Aitchison, and TW Macfarlane. "The value of the coefficient of variation in assessing repeat variation in ECG measurements." *Eur Heart J 19: 342-351*, 1998.

Medina, MA, et al. "Polymer therapy: a novel treatment to improve fat graft viability." *Plast Reconstr Surg 127(6): 2270-2282*, 2011.

Michoell, A, et al. "Influence of Porosity on the Electrical Sensing Zone and Laser Diffraction Sizing of Silicas. A Collaborative Study." *Part Part Syst Charact 11: 391-397*, 1994.

Mikkelsen, H, Z Duck, KS Lilley, and M Welch. "Interrelationships between Colonies, Biofilms, and Planktonic Cells of Pseudomonas aeruginosa." *J Bacteriol 189: 2411–2416*, 2007.

Millipore.

"http://www.millipore.com/userguides.nsf/a73664f9f981af8c852569b9005b4eee/0ae31d4097850fd285256bc30067a831/$FILE/P36198.pdf, accessed 17th May 2011." 2011.

Mittal, R, S Sharma, S Chhibber, and K Harjai. "Evaluation of tumour necrosis factor-alpha and interleukin-1beta in an experimental pyelonephritis model induced with planktonic and biofilms cells of Pseudomonas aeruginosa." *Can J Infect Dis Med Microbiol 20(3): e35-42*, 2009.

Moghimi, SM, and JC Murray. "Poloxamer-188 revisited: a potentially valuable immune modulator?" *J Nat Cancer Institut 88(11): 766-768*, 1996.

Moioli, EK, L Hong, J Guardado, PA Clark, and JJ Mao. "Sustained release of TGFbeta3 from PLGA microspheres and its effect on early osteogenic differentiation of human mesenchymal stem cells." *Tissue Eng 12(3): 537-546*, 2006.

Mordhorst, IL, et al. "O-acetyltransferase gene neuO is segregated according to phylogenetic background and contributes to environmental desiccation resistance in Escherichia coli K1." *Environ Microbiol 11(12): 3154-3165*, 2009.

Morishita, M, and K Park. *Biodrug delivery systems: Fundamentals, Applications and Clinical Development.* New York: Informa Healthcare, 2010.

MRC Multi-Centre Otitis Media Study Group. "Risk factors for persistence of bilateral otitis media with effusion." *Clin Otolaryngol 26: 147-156*, 2001.

MRC Multi-centre Otitis Media Study Group. "Surgery for persistent otitis media with effusion: generalisability of results from the UK trial (TARGET)." *Clin Otolaryngol*, 2001: 417-24.

Nguyen, CT, et al. "Noninvasive in vivo optical detection of biofilm in the human middle ear." *Proc Natl Acad Sci USA 109(24): 9529-9534*, 2012.

Nguyen, CT, H Tu, EJ Chaney, CN Stewart, and SA Boppart. "Non-invasive optical interferometry for the assessment of biofilm growth in the middle ear." *Biomed Opt Express 1(4): 1104-1116*, 2010.

NHS Centre for Reviews and Dissemination. "Implementing clinical guidelines: can guidelines be used to improve clinical practice?" *Effective Health Care 1(8): 1-12*, 1994.

NHS Centre for Reviews and Dissemination. "The treatment of persistent glue ear in children." *Effective Health Care 1(4): 1-16*, 1992.

NICE. National Institute for Health and Clinical Excellence: Surgical management of otitis media with effusion. 2008.

Nickel, JC, I Ruseska, JB Wright, and JW Costerton. "Tobramycin resistance of Pseudomonas aeruginosa *cells growing as a biofilm on urinary catheter* material." *Antimicrob Agents Chemother 27(4): 619-624*, 1985.

Ohta, S, et al. "Pluronic F127: application in arterial embolization." *J Vasc Interv Radiol 17(3): 533-539*, 2006.

Olofsson, SK, and O Cars. "Optimizing drug exposure to minimize selection of antibiotic resistance." *Clinical Infectious Diseases 45: S129–136*, 2007.

Olson, ME, H Ceri, DW Morck, AG Buret, and RR Read. "Biofilm bacteria: formation and comparative susceptibility to antibiotics." *Can J Vet Res 66: 86-92*, 2002.

O'Neill, AJ, K Miller, B Oliva, and I Chopra. "Comparison of assays for detection of agents causing membrane damage in Staphylococcus aureus." *J Antimicrob Chemother 54(6): 1127-1129*, 2004.

Otto, M. "Staphylococcal Biofilms." *Curr Top Microbiol Immunol*, 2008: 322: 207–228.

Ovesen, T, JU Felding, B Tommerup, LP Schousboe, and CG Petersen. "Effect of N-acetylcysteine on the incidence of recurrence of otitis media with effusion and re-insertion of ventilation tubes." *Acta Otolaryngol Suppl 543: 79-81*, 2000.

Oxman, AD, MA Thomson, DA Davis, and B Haynes. "No magic bullets: A systematic review of 102 trials of interventions to improve professional practice." *Can Med Assoc J 153: 1423–1431*, 1995.

Ozcan, C, Y Vayisoglu, F Otag, A Polat, K Gorur, and O Ismi. "Does Helicobacter pylori have a role in the development of chronic otitis media with effusion? A preliminary study." *J Otolaryngol Head Neck Surg 38(5): 526-531*, 2009.

Palmer, RJ, PI Diaz, and PE Kolenbrander. "Rapid Succession within the Veillonella Population of a Developing Human Oral Biofilm In Situ." *J Bacteriol*, 2006: 188: 4117–4124.

Paluch-Oleśa, J, et al. "The phenotypic and genetic biofilm formation characteristics of coagulase-negative staphylococci isolates in children with otitis media." *Int J Pediat Otorhinolaryngol 75(1): 126-130* , 2011.

Paradise, JL, et al. "Tympanostomy tubes and developmental outcomes at 9 to 11 years of age." *N Engl J Med 356(3): 248-61*, 2007.

Parkinson, N, et al. "Mutation at the Evi1 locus in Junbo mice causes susceptibility to otitis media." *PLoS Genet 2(10): e149*, 2006.

Parsek, MR, and PK Singh. "Bacterial biofilms: An emerging link to disease pathogenesis." *Annu Rev Microbiol*, 2003: 57:677–701.

Peizhong, L, K Whatmough, JP Birchall, JA Wilson, and JP Pearson. "Does the bacterial DNA found in middle ear effusions come from viable bacteria?" *Clin Otolaryngol 25(6): 570-576*, 2000.

Perletti, G, et al. "Macrolides for the treatment of chronic bacterial prostatitis: an effective application of their unique pharmacokinetic and pharmacodynamic profile." *Mol Med Report 4(6): 1035-1044*, 2011.

Perron, JK, HE Naguib, J Daka, A Chawla, and R Wilkins. "A study on the effect of degradation media on the physical and mechanical properties of porous PLGA 85/15 scaffolds." *J Biomed Mater Res B Appl Biomater 91(2): 876-886*, 2009.

Pietrzak, WS. "Effects of ethylene oxide sterilization on 82: 18 PLLA/PGA copolymer craniofacial fixation plates." *J Craniofac Surg 21(1): 177-181*, 2010.

Poetker, DM, DR Lindstrom, CE Edmiston, CJ Krepel, TR Link, and JE Kerschner. "Microbiology of middle ear effusions from 292 patients undergoing tympanostomy tube placement for middle ear disease." *Int J Pediatr Otorhinolaryngol 69(6): 799-804*, 2005.

Popova, D, S Varbanova, and TM Popov. "Comparison between myringotomy and tympanostomy tubes in combination with adenoidectomy in 3-7-year-old children with otitis media with effusion." *Int J Pediatr Otorhinolaryngol 74(7): 777-780*, 2010.

Post, JC, et al. "Molecular analysis of bacterial pathogens in otitis media with effusion." *JAMA 273(20): 1598-1604*, 1995.

Proctor, R, and A von Humboldt. "Bacterial energetics and antimicrobial resistance." *Drug Resist Updat 1(4): 227-235*, 1998.

Proctor, RA, et al. "Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections." *Nat Rev Microbiol 4(4): 295-305*, 2006.

Ranade, VV, and MA Hollinger. *Drug delivery systems.* Boca Raton: CRC press, 2003.

Rayner, MG, Y Zhang, MC Gorry, Y Chen, JC Post, and GD Ehrlich. "Evidence of bacterial metabolic activity in culture-negative otitis media with effusion." *JAMA 279(4): 296-299*, 1998.

Reeves, R, A Ribeiro, L Lombardo, R Boyer, and JB Leach. "Synthesis and Characterization of Carboxymethylcellulose- Methacrylate Hydrogel Cell Scaffolds." *Polymers 2: 252-264*, 2010.

Reinhart, CT, RW Korsmeyer, and NA Peppas. "Macromolecular network structure and its effect on drug and protein diffusion." *Int J Pharm Tech Prod Mfr 2(2): 9*, 1981.

Rosenfeld, RM, et al. "Clinical practice guideline: Otitis media with effusion." *Otolaryngol Head Neck Surg 130 (5 Suppl): S95-118*, 2004.

Rosenfeldt, RM, and CD Bluestone. *Evidence-based otitis media*. Hamilton: BC Decker Inc, 1999.

Rosoff, M. *Controlled release of drugs: polymers and aggregate systems*. New York: VCH Publishers, 1989.

Rovers, MM, AG Schilder, GA Zielhuis, and RM Rosenfeld. "Otitis media." *Lancet 363(9407): 465-473*, 2004.

Sachidanandham, R, KY Gin, and CL Poh. "Monitoring of active but non-culturable bacterial cells by flow cytometry." *Biotechnol Bioeng 89(1): 24-31*, 2005.

Sade, J, and Z Weissman. "Middle ear mucosa and secretory otitis media." *Arch Otorhinolaryngol 215(3-4): 195-205*, 1977.

Saito, T, et al. "Prevention of persistent ear drum perforation after long-term ventilation tube treatment for otitis media with effusion in children." *Int J Pediatr Otorhinolaryngol 38(1): 31-39*, 1996.

Sakamoto, T, et al. "Inner ear drug delivery system from the clinical point of view." *Acta Otolaryngol Suppl 563: 101-104*, 2010.

Saleem, I, MJ Donovan, and HDC Smyth. "Particle Size Analysis: from Microparticles to Nanosystems." *Am Pharmaceut Rev 10(2): 126-131*, 2007.

Saltzman, WM. *Drug delivery: engineering principles for drug therapy.* Oxford: Oxford University Press, 2001.

Saukkoriipi, A, A Palmu, T Kilpi, and M Leinonen. "Real-time quantitative PCR for the detection of Streptococcus pneumoniae in the middle ear fluid of children with acute otitis media." *Mol Cell Probes*, 2002: 16(5): 385-90.

Schilder, AG, W Lok, and MM Rovers. "International perspectives on management of acute otitis media: a qualitative review." *Int J Pediatr Otorhinolaryngol 68: 29-36*, 2004.

Serra, L, J Domenech, and NA Peppas. "Engineering design and molecular dynamics of mucoadhesive drug delivery systems as targeting agents." *Eur J Pharmaceutics Biopharmaceutics 71: 519–528*, 2009.

Shearer, H, MJ Ellis, SP Perera, and JB Chaudhuri. "Effects of common sterilization methods on the structure and properties of poly(D,L lactic-co-glycolic acid) scaffolds." *Tissue Eng 12(10): 2717-2727*, 2006.

Shen, Y, B Mei Teh, PL Friedland, RH Eikelboom, and MD Atlas. "To pack or not to pack? A conteporary review of middle ear packing agents." *Laryngoscope 121(5): 1040-1048*, 2011.

Shinkwin, CA, GE Murty, R Simo, and NS Jones. "Per-operative antibiotic/steroid prophylaxis of tympanostomy tube otorrhoea: chemical or mechanical effect?" *J Laryngol Otol 110(6): 531-533*, 1996.

Sihorkar, V, and SP Vyas. "Biofilm Consortia on Biomedical and Biological Surfaces: Delivery and Targeting Strategies." *Pharmaceut Res 18(9): 1247-1254*, 2001.

Silverson. "www.silverson.com. Accessed 19th October 2010." 2010.

Simoes, S, A Figueiras, and F Veiga. "Modular Hydrogels for Drug Delivery." *Journal of Biomaterials and Nanobiotechnology 3: 185-199*, 2012.

Singh-Joy, SD, and VC McLain. "Safety assessment of poloxamers as used in cosmetics." *Int J Toxicol 27 Suppl 2: 93-128*, 2008.

Smirnova, MG, SL Kiselev, NV Gnuchev, JP Birchall, and JP Pearson. "Role of the pro-inflammatory cytokines tumor necrosis factor-alpha, interleukin-1beta, interleukin-6 and interleukin-8 in the pathogenesis of the otitis media with effusion." *Eur Cytokine Netw 13(2): 161-172*, 2002.

Smith, AW. "Biofilms and antibiotic therapy: is there a role for combating bacterial resistance by the use of novel drug delivery systems?" *Adv Drug Deliv Rev 57(10): 1539-1550*, 2005.

Song, JJ, JD Lee, BD Lee, SW Chae, and MK Park. "Effect of diesel exhaust particles on human middle ear epithelial cells." *Int J Ped Otorhinolaryngol 76: 334-338*, 2012.

Souli, M, and H Giamarellou. "Effects of slime produced by clinical isolates of coagulase-negative staphylococci on activities of various antimicrobial agents." *Antimicrob Agents Chemother 42(4): 939-941*, 1998.

Starner, TD, N Zhang, GH Kim, MA Apicella, and PB McCray Jr. "Haemophilus influenzae forms biofilms on airway epithelia." *Am J Respir Crit Care Med*, 2006: 174: 213–220.

Steinke, JW, CR Woodard, and L Borish. "Role of hypoxia in inflammatory upper airway disease." *Curr Opin Allergy Clin Immunol 8(1): 16-20*, 2008.

Stevens, J. "Post Hoc Tests in ANOVA." *http://pages.uoregon.edu/stevensj/posthoc.pdf, accessed 11th July 2011*, 1999.

Stinner, DJ, SP Noel, WO Haggard, JT Watson, and JC Wenke. "Local antibiotic delivery using tailorable chitosan sponges: the future of infection control?" *J Orthop Trauma 24(9): 592-597*, 2010.

Sun, Y, et al. "Biofilm formation and Toll-like receptor 2, Toll-like receptor 4, and NF-kappaB expression in sinus tissues of patients with chronic rhinosinusitis." *Am J Rhinol Allergy 26(2): 104-109*, 2012.

Sundberg, L, T Edén, S Ernstson, and R Pahlitzsch. "Penetration of erythromycin through respiratory mucosa. A study using secretory otitis media as a model." *Acta Otolaryngol Suppl 365: 1-17*, 1979.

Svenson, S. *Carrier-based drug delivery.* Washington: American Chemical Society, 2004.

Swidsinski, A, et al. "Comparative study of the intestinal mucus barrier in normal and inflamed colon." *Gut 56(3): 343-350*, 2007.

Tao, Y, Y Lu, Y Sun, B Gu, W Lu, and J Pan. "Development of mucoadhesive microspheres of acyclovir with enhanced bioavailability." *Int J Pharmaceutics 378: (30-36)*, 2009.

Thomas, RG, C Ojano-Dirain, and PJ Antonelli. "Topical antibiotic treatment reduces tympanostomy tube biofilm formation." *Laryngoscope 121: 1067-1071*, 2011.

Thorn, V. "Tissue concentrations of ofloxacin in the middle ear." *Clin Ther 9(5): 523-527*, 1987.

Thornton, RB, et al. "Multi-species bacterial biofilms and intracellular infection in otitis media." *BMC Pediatr 11: 94*, 2011.

Tirumalai, RS. "Revised sterility tests and sterility assurance." *http://www.usp.org/pdf/EN/meetings/asMeetingIndia2009/session2Track2Tirumalai_2.pdf, accesssed 17th May 2011*, 2009.

Tonnaer, EL, EA Mylanus, JJ Mulder, and JH Curfs. "Detection of bacteria in healthy middle ears during cochlear implantation." *Arch Otolaryngol Head Neck Surg 135(3): 232-237*, 2009.

Tos, M, and K Bak-Pedersen. "Goblet cell population in the normal middle ear and Eustachian tube of children and adults." *Ann Otol Rhinol Laryngol 85(2 Suppl 25 Pt 2): 44-50*, 1976.

Tsukada, Y, et al. "Partcile size control of poly(dl-lactide-co-glycolide) nanospheres for sterile applications." *Int J Pharm 31; 370(1-2): 196-201*, 2009.

Uchegbu, IF, and AG Schatzlein. *Polymers in drug delivery*. Boca Raton: Taylor & Francis, 2006.

van den Aardweg, MTA, AGM Schilder, E Herkert, CWB Boonacker, and MM Rovers. "Adenoidectomy for otitis media in children (Review)." *Cochrane Database of Systematic Reviews*, 2010.

van der Waaij, LA, et al. "Bacterial population analysis of human colon and terminal ileum biopsies with 16S rRNA-based fluorescent probes: commensal bacteria live in suspension and have no direct contact with epithelial cells." *Inflamm Bowel Dis 11(10): 865-871*, 2005.

Vorachit, M, K Lam, P Jayanetra, and JW Costerton. "Resistance of Pseudomonas pseudomallei growing as a biofilm on silastic discs to ceftazidime and co-trimoxazole." *Antimicrob Agent Chemoth 37(9): 2000-2002.*, 1993.

Wang, X, et al. "Dose-dependent sustained release of dexamethasone in inner ear cochlear fluids using a novel local delivery approach." *Audiol Neurootol 14(6): 393-401*, 2009.

Wang, X, L Dellamary, R Fernandez, Q Ye, C LeBel, and F Piu. "Principles of inner ear sustained release following intratympanic administration." *Laryngoscope 121: 385–391*, 2011.

West, LJ. "Defining critical windows in the development of the human immune system." *Hum Exp Toxicol 21: 499-505*, 2002.

Wickhamlabs. *http://www.wickhamlabs.co.uk/microbiology_medicaldevice/services.html, accessed 17th May 2011*, 2011.

Wu, YT, H Zhu, M Willcox, and F Stapleton. "Removal of biofilm from contact lens storage cases." *Invest Ophthalmol Vis Sci. Epub ahead of print*, 2010.

Xu, L, et al. "Role of the luxS Quorum-Sensing System in Biofilm Formation and Virulence of Staphylococcus epidermidis." *Infect Immunity 74: 488-496*, 2006.

Xu, R, and OA Di Guida. "Comparison of sizing small particles using different technologies." *Powder Technology 132: 145-153*, 2003.

Yaman, H, S Yilmaz, N Alkan, B Subasi, E Guclu, and O Ozturk. "Shepard grommet tympanostomy tube complications in children with chronic otitis media with effusion." *Eur Arch Otorhinolaryngol 267(8): 1221-1224*, 2010.

Yasuda, H, CE Lamaze, and ID Ikenberry. "Permeability of solutes through hydrated polymer membranes. part I. Diffusion of sodium chloride." *Makromol Chem 118: 19*, 1968.

Yilmaz, T, Ceylan, Akyon, Y M, O Ozcakyr, and B Gursel. "Helicobacter pylori: a possible association with otitis media with effusion." *Otolaryngol Head Neck Surg 134(5): 772-777*, 2006.

Zhang, Y, et al. "A novel paclitaxel-loaded poly(epsilon-caprolactone)/Poloxamer 188 blend nanoparticle overcoming multidrug resistance for cancer treatment." *Acta Biomater 6(6): 2045-2052*, 2010.

Zhou, Z, L Chen, P Liu, M Shen, and F Zou. "Simultaneous Determination of Isoniazid, Pyrazinamide, Rifampicin and Acetylisoniazid in Human Plasma by High-Performance Liquid Chromatography." *Analytical Sciences 26: 1133-1138*, 2010.