An alternative to animal experiments: Development of an *in vitro* human skin model for evaluation of topical antimicrobial compounds

Project funded by the Danish 3R-Center

Contact person and Principal Investigator Ph.D. Mette E. Skindersø
Statens Serum Institut/Chr. Hansen A/S
Research Assistant M. Sc. June L. Hansen
Rigshospitalet, Department of Clinical Microbiology

October 2017

Dansk resumé

I Danmark anvendes der mindst 500 forsøgsmus om året alene til hudinfektionsstudier, hvilket omregnet for hele EU svarer til omkring 25.000 mus. Cytotoxicitet og allergenicitet af topikale lægemidler kan i dag testes ved anvendelse af human *in vitro* hud dyrket i en Petri-skål, hvorimod virkningsgraden af medicin mod hudinfektioner på nuværende tidspunkt kun kan testes ved anvendelse af forsøgsdyr, da der ikke eksisterer en *in vitro* model til dette. Formålet med projektet har derfor været at udvikle og etablere en model baseret på kunstig hud, der kan anvendes som alternativ til dyreforsøg i forbindelse med forskning i sår-infektioner.

Denne *in vitro* sår-infektionsmodel er baseret på kommercielt tilgængelig fuldtudviklet *in vitro*-dyrket human hud (EpiDermFT, MatTek). Den kunstige hud er påført et biopsi-sår (3 mm i diameter), der inficeres med stafylokokker eller andre relevante bakterier. Efter infektionen er etableret (18 timer) påbegyndes behandling med topikal antimikrobiel salve to gange dagligt.
Vi har anvendt denne model til at teste virkningsgraden af to meget anvendte topikale lægemidler (mupirocin og fusidinsyre) til behandling af infektion med Methicillin Resistent *Staphylococcus aureus* (MRSA). Desuden har vi undersøgt effekten af såvel infektion som behandling af infektion (med mupirocin- og fusidinsyre-salve) ved at måle niveauet af 29 cytokiner og kemokiner. Hudstykkerne er desuden undersøgt ved histologi.

Bakteriemængden i sår behandlet med fusidinsyre eller mupirocin var henholdvis 10.000 gange og 200.000 gange lavere end i ubehandlede inficerede sår, hvilket er sammenligneligt med data fra *in vivo* museforsøg, hvor samme topikale lægemidler er blevet undersøgt. Vi fandt desuden et markant fald i inflammations-niveauet for de behandlede sår i forhold til ubehandlede sår baseret på cytokin og kemokin-profilerne. Histologien viste, at de tilbageværende bakterier i de behandlede sår primært var lokaliseret i sårranden, hvor de måske er fysisk beskyttet mod behandlingen med fusidinsyre eller mupirocin pga. små revner i huden.

Vi brugte desuden infektionsmodellen til at undersøge infektionsgraden af tre forskellige stafylokokker (uden behandling). Vi fandt at *S. pseudintermedius*, en opportunistisk patogen som ofte findes hos husdyr, inficerede huden i næsten ligeså høj grad som de to *S. aureus* stammer (en methicillin sensitiv og en methicillin resistent stamme), dog forårsagede *S. pseudintermedius* stammen et lavere inflammationsniveau (baseret på cytokin- og kemokin-profilen) end de to (human patogene) *S. aureus* stammer. Forskellene mellem de to *S. aureus* stammer var små (ikke signifikante).

Ovenstående viser, at *in vitro* sår-infektionsmodellen udviklet i dette projekt kan anvendes til at undersøge niveauet af bakterier i inficerede sår med eller uden behandling, endvidere kan modellen bruges til at undersøge ændringer i cytokinprofil og histologi forårsaget af infektion og/eller behandling. Modellen kan desuden anvendes til at sammenligne infektion med forskellige stammer (evt. mutanter) af bakterier.
Således tilbyder modellen et velegnet alternativ til dyremodeller for forskning i sår-infektioner, herunder, men ikke begrænset til, forskning i effekten af topikale antimikrobielle stoffer.

Resume (english)

In Denmark, at least 500 mice per year are used for skin infection studies, corresponding to around 25,000 mice throughout EU. *In vitro* skin models, such as artificial human skin grown in a Petri dish, are used to test cytotoxicity and allergenicity of topical drugs. However, the efficacy of antimicrobial drugs for skin infections are currently tested *in vivo*, as no *in vitro* model for this purpose exists. Thus, the aim of this project has been to develop, implement and evaluate an *in vitro* skin infection model for use as an alternative to animal models for test of the efficacy of topical antimicrobial compounds and investigation of bacterial load by different pathogens.

The *in vitro* wound infection model is based on commercially available *in vitro* skin (EpiDerm-FT, MatTek). The skin consists of epidermal keratinocytes and dermal fibroblasts, which have been cultured at the air/liquid interface to form a multilayered model of the human skin, including a fully developed basement membrane. A biopsy punch is used to expose the dermis while leaving the basement membrane intact, thereby resembling a superficial skin wound (3 mm in diameter). Then the wound is infected with bacteria, and the infection is allowed to develop (18 h), whereupon topical treatment with antimicrobial ointment is performed twice daily.

We have used the model for evaluation of treatment efficacy of two frequently used topical antimicrobials; fusidic acid and mupirocin for treatment of infection with Methicillin Resistant *Staphylococcus aureus* (MRSA). We have also measured the effect of infection and treatment of the infection upon the level of 29 cytokines and chemokines, and the skin pieces has been subjected to histology.

The level of bacteria in the wounds treated with fusidic acid and mupirocin were, respectively, 10.000 and 200.000 lower than in untreated infected wounds. This treatment effect is comparable to what is found using an *in vivo* mouse wound infection model investigating the same two topical antimicrobials. The cytokine and chemokine profile indicated a markedly lower inflammation level for the infected wounds treated with the two antimicrobials than for the untreated infected wounds. The histology showed that the bacteria remaining in the treated wounds were primarily located in the wound periphery, maybe because the bacteria here are protected against the treatment by small cracks in the skin.

We also used the wound infection model to investigate the infection level (without treatment) of three different Staphylococcus strains. We found that *S. pseudintermedius* (an opportunistic pathogen of domestic animals) infected the skin model almost to the same level as the two *S. aureus* strains (one methicillin sensitive and one methicillin resistant strain), however the *S. pseudintermedius* caused a lower inflammation level (based on the cytokine and chemokine profile) than the two *S. aureus* strains. Infection level and cytokine and chemokine profile were not significantly different for the two *S. aureus* strains.

Thus we show here the *in vitro* wound infection model developed can be used for examining the bacterial load with or without treatment with topical antimicrobials. Moreover, the model can be used to examine the bacterial load of different strains of bacteria. Investigation of infections based on histology and cytokine profile can also be performed using the *in vitro* wound infection model. Thus the model offers a strong alternative to animal models for research in wound infections, including but not limited to investigations of the efficacy of topical antimicrobial compounds.

Table of contents

Introduction

Animal models are used worldwide to investigate biological responses and novel medical therapies, such as wound healing, infections and antimicrobials. In 2015, 241,657 animals were used for experimental procedures in Denmark alone[1]. Although it is likely not possible to eliminate all animal testing, it may be possible to reduce the number by replacing animals with alternative models especially in the initial phase of research for example by using human cells grown in microtiter plates.

Animal models are commonly used to evaluate novel medical therapies and the two crucial steps during drug development are assessment of toxicity and validation of efficacy.
While *in vitro* cytotoxicity assays for testing topical drugs are used worldwide, the antibacterial efficacy towards wound infections currently has to be tested *in vivo*, as no *in vitro* model for this purpose exists. An *in vitro* wound infection model would greatly benefit development of novel topical antimicrobials for human as well as veterinary use. Especially with the increasing resistance and multi-resistance towards antimicrobials in several bacterial species, new antimicrobial compounds are needed. In addition, an *in vitro* infection model would be useful for investigating pathogenicity and virulence of bacterial strains especially for research on genetically modified bacteria (e.g. knock-out mutants), as work with Gene Modified Organisms in animal facilities are difficult due to strict regulations and requirements.

Staphylococcus aureus is a major cause of skin infections and infections caused by methicillin-resistant *S. aureus* (MRSA) is increasing in Denmark; the number of humans reported to be infected with MRSA has increased from 661 in 2007 to 3,551 in 2016 (Number of reported cases to Statens Serum Institut[2]).

We here report on the evaluation of an *in vitro* skin model, as an alternative to animal models for research on wound infections. A 3D full thickness human skin model (EpiDermFT) from MatTek (Ashland, USA), consisting of dermal fibroblast and epidermal keratinocytes, inflicted with a biopsy wound was infected with staphylococci. The model was used to evaluate efficacy of two topical ointments; fusidic acid and mupirocin, which are commonly used to treat staphylococcal infections in Denmark. In addition we used the model to evaluate the virulence of different strains belonging to the genus *Staphylococcus.*

Materials and methods

Evaluation of topical treatment of methicillin-resistant Staphylococcus aureus using the in vitro skin wound model

The *in vitro* wound infection model is an adaptation of the murine wound infection model developed by Lundberg and Frimodt-Møller[3]. EpiDermFT from MatTek (Ashland, USA) inflicted with biopsy wounds (3 mm in diameter) were grown and maintained in antibiotic free media. Skin pieces were infected with *S. aureus* 43484 CA-MRSA, and the infection was allowed to establish for 18 hours. Then the infected wounds were treated with topical antimicrobial ointment, Fucidin® 2% (fusidic acid, LEO Pharma A/S, Ballerup, Denmark) and Bactroban® 2% (mupirocin, GlaxoSmithKline Pharma A/S, Brøndby Denmark) twice daily or left untreated. Supernatants were collected, sterile filtered and frozen for cytokine analysis (Section 0). Skin pieces were sampled according to the table below. The pieces were cut into halves. Half of the skin pieces were subjected for histology and the other half used for CFU (Colony Forming Unit) analysis.

	Day(s) after skin received	**Day(s) after infection**	**Day(s) after 1st treatment**	**Uninfected**	**CA-MRSA**			**MSSA PVL-**
				Untreated	**Untreated**	**Mupirocin**	**Fusidic acid**	**Untreated**
Day 0 (in fridge)	0	-	-					
Day 1 infection	1	Day 1 infection	-					
Day 2 1st treatment	2	Day 2 infection	Day 1 treatment	1	2			2
Day 3	3	Day 3 infection	Day 2 treatment					
Day 4	4	Day 4 infection	Day 3 treatment	1	6	6	6	
Total				2	8	6	6	2

Evaluation of bacterial load for different Staphylococcal strains using the *in vitro* skin model

EpiDermFT from MatTek (Ashland, USA) skin pieces with 3 mm biopsy wounds were grown in antibiotic free media. Skin pieces were infected with one of the following staphylococci; *S. aureus* 43484 (CA-MRSA t008 PVL+), *S. aureus* 114864 (MSSA t008 PVL-) or *S. pseudintermedius* DK729 (methicillin resistant *S. pseudintermedius*, MRSP) or left uninfected for control. Supernatants were collected, sterile filtered and frozen for cytokine analysis.

Skin pieces were sampled according to the table below. The pieces were cut into halves. Half of the skin pieces are subjected for histology and the other half used for CFU analysis.

Day(s) after skin received	Day(s) after infection	Uninfected	43484 CA-MRSA t008 PVL+	114864 MSSA t008 PVL-	DK729 MRSP
Day 0 equilibrate skin	-				
Day 1 infection	Day 0 infection				
Day 2	24 h post infection				
Day 3	48 h post infection	2	4	4	4
Day 4	72 h post infection				
Day 5	96 h post infection	2	2	2	
Total		4	6	6	4

Cytokine analysis of supernatants from *in vitro* skin infected with staphylococci

The cytokine level were measured in supernatants collected from Evaluation of topical treatment of methicillin-resistant Staphylococcus *aureus using* the in vitro skin wound model and from Evaluation of bacterial load *for different* Staphylococcal strains using the *in vitro* skin model.

V-PLEX Human Cytokine 30-Plex Kit from Mesoscale (modified to a 29-Plex kit as the MCP-4 assay component of the kit could not be delivered) was used to measure the cytokine and chemokine levels.

Cytokines and chemokines were measured according to manufacturer's recommendations.

Results and discussion

Topical treatment of methicillin-resistant *Staphylococcus aureus* in an *in vitro* skin wound infection model

The *in vitro* wound infection model were infected with CA-MRSA and treated with one of two types of antimicrobial ointment either fusidic acid (Fucidin®) or mupirocin (Bactroban®) or left untreated for control. The amount of bacteria in untreated *in vitro* wounds were determined to $7 \cdot 10^7$ CFU pr. wound at day 1 after infection and $2 \cdot 10^9$ CFU per wound at day 3 after infection (Figure 1A), i.e. the bacterial load increased 30 times from day 1 to day 3. For comparison, Lundberg and Frimodt-Møller, 2013[3] found a 10 times increase in bacterial load from day 1 to day 4 for infected wounds *in vivo* (mice) (Figure 2A[3]).

Treatment with antimicrobial ointment resulted in a significant reduction in bacterial numbers per wound compared with untreated, infected *in vitro* wounds (Figure 1B). At day 3 the treatment with fusidic acid caused an approximately 10,000 times reduction in the level of bacteria compared to untreated, infected wounds. At day 3, the level of bacteria in the wounds treated with mupirocin was approximately 200,000 times lower than in untreated wounds. This is comparable to the reduction found by Lundberg and Frimodt-Møller, 2013 *in vivo* (mice), where mupirocin also was shown to be more effective than fusidic acid at day 6 (Figure 2B+C).

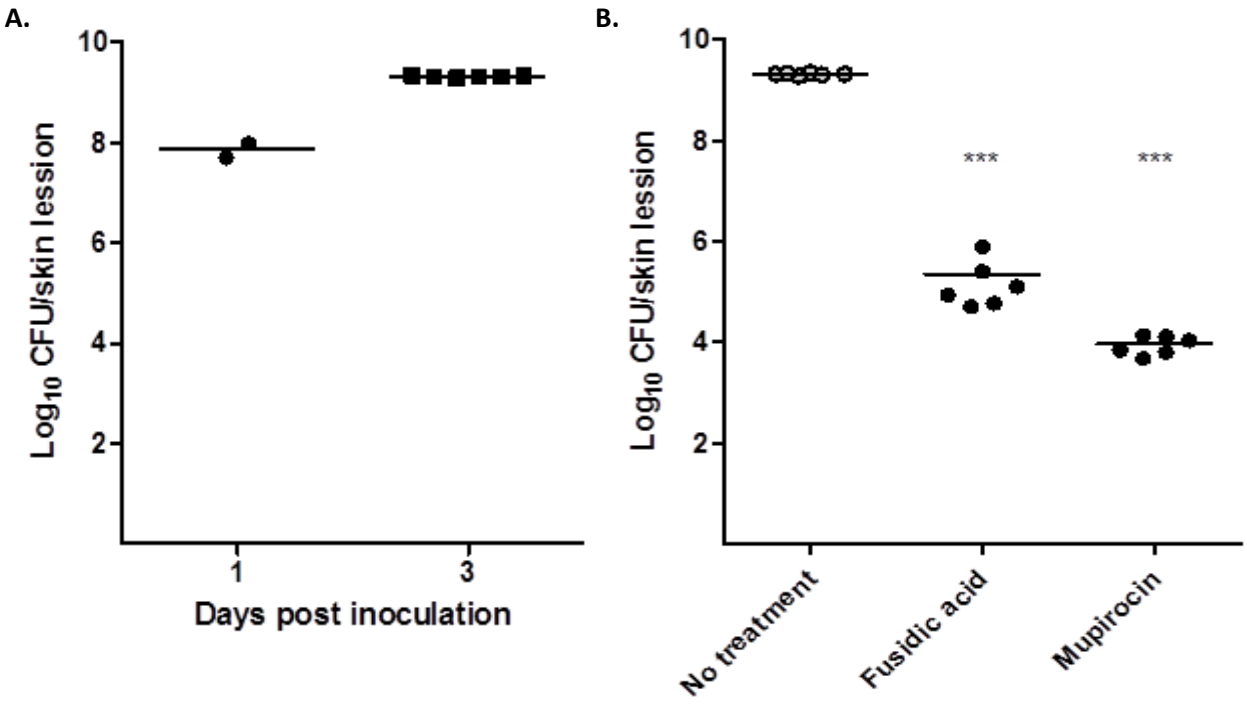


Figure 1 *In vitro* wounds in artificial skin infected with *S. aureus* (CA-MRSA) 43484. The wounds were infected with $1 \cdot 10^6$ CFU per wound. The amount of bacteria per *in vitro* skin piece was determined at day 1 and 3 post infection. **A.** CFU per untreated, infected *in vitro* skin at day 1 and day 3. **B.** The effect of the topical treatment with antibiotic ointment at day 3. The skin were treated twice daily with fusidic acid or mupirocin for two days. No treatment = untreated. The bar shows the geometric mean. *** Statistical significant difference ($P < 0.001$) between treated and untreated groups (**B**).

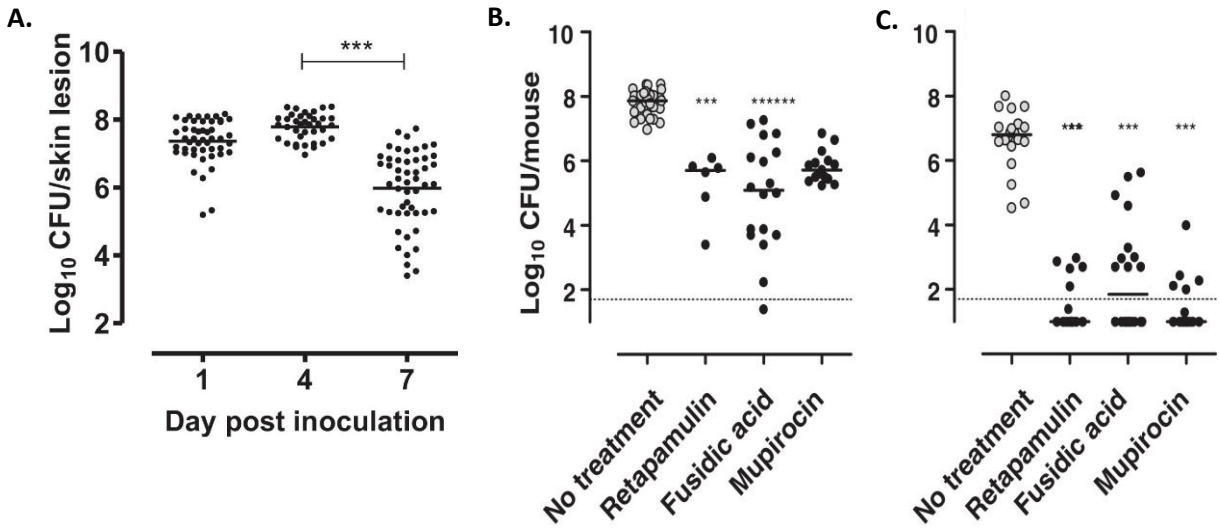


Figure 2 Superficial wounds on mice infected with *S. aureus* (CA-MRSA) 43484. Figure from article by Lundberg and Frimodt-Møller, 2013[3] included for comparison. Wounds were infected with $1{\cdot}10^7$ CFU. **A.** The amount of bacteria per wound was determined at day 1, 4 and 7 post infection. Data compiled from four studies with similar results (9–12 mice/group in each of the four studies). The wounds were treated twice a day with retapamulin, fusidic acid or mupirocin for (**B**) 3 and (**C**) 6 days. The total amount of bacteria per wound was determined the day after last treatment. Bars show the geometric mean. *** Statistically significant difference ($P < 0.001$) between groups compared with each other (**A**) or between untreated group vs treated groups (**B** & **C**). The dotted line represents the limit of detection. Data compiled from three studies with similar results (6–8 mice/treatment group in each of the three studies). Retapamulin treatment was only included in one of the three studies in (**B**) and in two of the three studies in (C), and mupirocin treatment was only included in two of the three studies in (**C**).

Figure 3 (next page) shows histology sections of four *in vitro* wounds at day 3 post infection with *S aureus* (CA-MRSA). The histology shows that the bacteria (appearing as dark purple areas in the histology images) primarily adhered to the edge of the biopsy (Figure 3BCD). Apparently MatTek, the manufacturer of the *in vitro* skin, has accidently made small cracks in the dermis (fibroblast layer) when they created the wounds using a biopsy needle. Débris from the wound edge may also have made this area more favourable to the bacteria. The bacteria localized between epidermis (keratinocyte layer) and dermis and the bacteria localised deeply into the dermis at the wound edge may have been completely or partially protected from the action of the antimicrobial ointments (Figure 3CD).

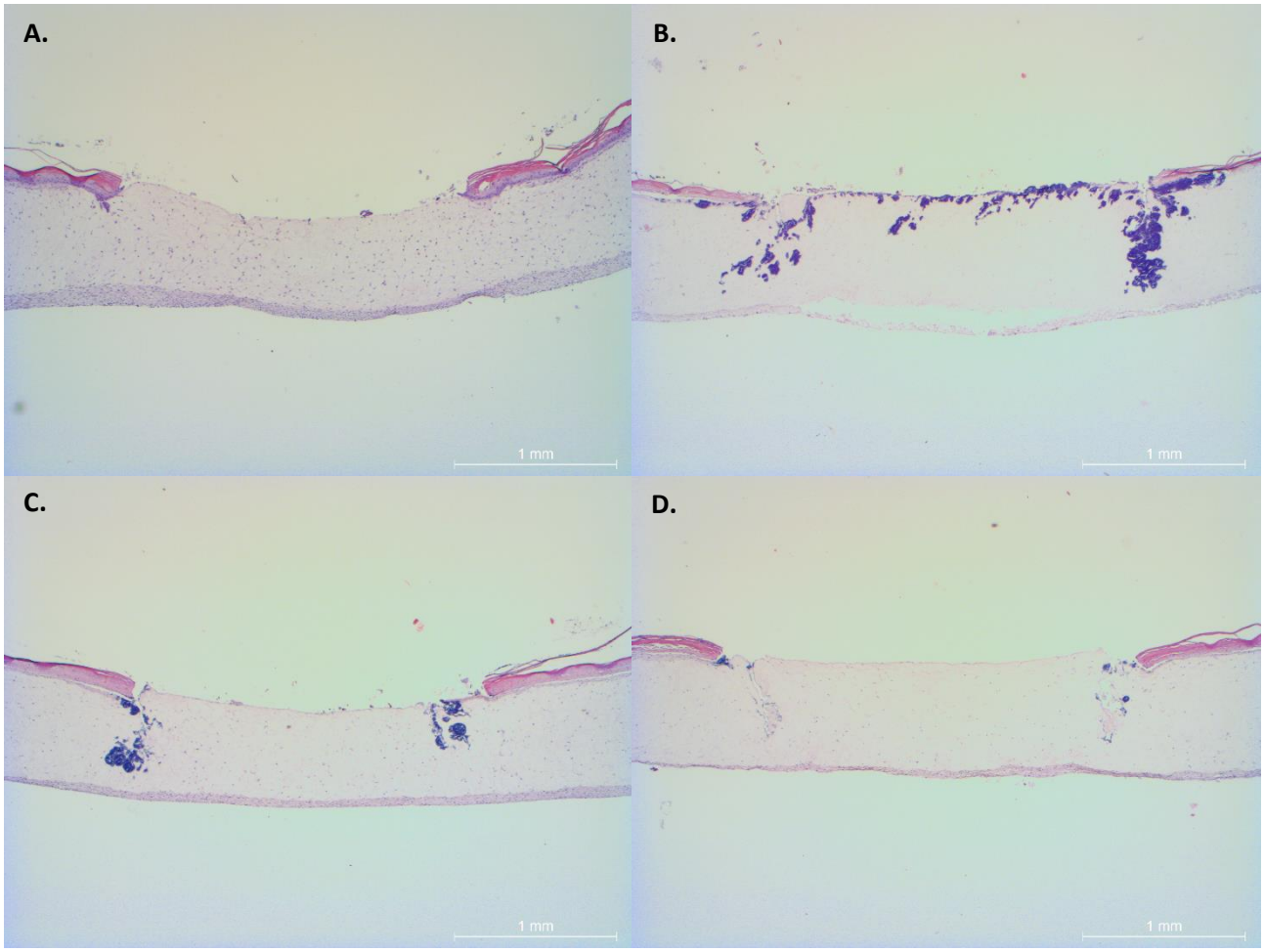


Figure 3 Histology sections of the *in vitro* wounds at day 3 post infection. **A**. Uninfected and untreated. **B**. Infected and untreated. **C**. Infected and treated with fusidic acid for 2 days. **D**. Infected and treated with mupirocin for 2 days.

The concentration of 29 cytokines in the supernatants (spent media) were measured and of these were 20 within (Table 1 and Table 2), 4 above (Table 3) and 5 below (Table 4) the measurable range of the assay kit.

Treatment of the infected wounds with fusidic acid and mupirocin caused a significant increase in the IL-10 level and significantly decreased the levels of GM-CFS and IL-1β measured in the supernatant (Table 1). IL-10 is an anti-inflammatory cytokine that regulates and limits the host immune response to pathogens hereby preventing damage to the host and maintaining homeostasis of normal tissue[4]. It inhibits synthesis of proinflammatory cytokines such as IFN-γ, IL-1β, IL-6, IL-8 IL-12, GM-CSF and TNF[5]. Thus, the profile seen with increased IL-10 and reduced GM-CFS and IL-1β suggests that the inflammation (caused by the infection) is resolved in response to treatment with antimicrobials and the lower bacterial load. Other proinflammatory cytokines such as IL-1α, IL-2 and IL-15 also decreased significantly in response to treatment with antimicrobials and resolution of the infection, while only insignificant to no decrease were measured for other proinflammatory cytokines such as IL-4, IL-6, IL12p70 and TNF-α (Table 1-Table 3).

Chemokines is a special family of cytokines that guides immune cells (macrophages, monocytes, neutrophils, dendritic cells etc.) to areas where they are needed e.g. due to inflammation. The release of

chemokines is often induced by proinflammatory cytokines[5]. Chemokines such as MIP-1α, MIP-1β, MDC-1, IL-8, IP-10 and Eotaxin-1 are produced during infection or injury and are known as inflammatory chemokines[6]. There was a significant increase in the concentration of Eotaxin-1 and MIP-1α in supernatants from wounds treated with fusidic acid compared to no treatment or to treatment with mupirocin (significance between groups by Tukey's post hoc analysis and Table 1). This suggests that fusidic acid and/or some of the other components in the ointment caused an increased response. Eotaxin-1 recruits eosinophils, which mediates allergic inflammation. To investigate whether fusidic acid induces an allergic reaction in the *in vitro* skin model, it may be relevant to investigate the effect of fusidic acid ointment on uninfected skin pieces in future studies.

Table 1 Levels of cytokines at day 3 (66 hours post infection) with significant differences between treated vs. untreated wounds. Ns (non-significant), * (P<0.05), ** (P<0.01) and *** (P<0.001). ↑/↓ Cytokine level for treated wounds is significantly higher/lower than for untreated wounds (one-way ANOVA with Dunnett's post-hoc analysis).

Cytokine	Uninfected	No treatment	Fusidic acid		Mupirocin	
	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Significance (no treatment vs. treatment)	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Significance (no treatment vs. treatment)
Eotaxin-1	252.0	915.5	1650.4	*** ↑	709.5	Ns
IL-7	2.7	0.6	1.0	*** ↑	1.2	*** ↑
IL-10	4.1	4.4	7.3	** ↑	6.6	** ↑
IP-10	66.2	19.6	34.3	*** ↑[1]	21.9	* ↑[1]
MIP-1α	20.6	42.2	55.9	** ↑	40.0	Ns
MIP-1β	4.3	6.5	7.9	* ↑	6.8	Ns
TARC	11.8	11.6	13.3	Ns	17.0	* ↑
VEGF-A	271.0	290.1	175.9[1]	* ↓	398.3	* ↑
Eotaxin-3	12.5	55.5	57.6	Ns	51.1	* ↓
GM-CSF	58.0	899.5	272.3	*** ↓	247.9	*** ↓
IL-1α	2.2	231.7	36.7	*** ↓	17.5	*** ↓
IL-1β	1.6	21.3	7.8	*** ↓[1]	6.3	*** ↓[1]
IL-2	2.3	9.3	8.0	Ns	7.3	* ↓
IL-13	11.2	29.9	27.3	** ↓	26.1	*** ↓
IL-15	2.4	5.2	3.2	*** ↓	2.5	*** ↓
TNF-α	5.4	18.9	16.8	Ns	15.1	** ↓

[1] One datapoint (outlier) was excluded from the calculation.

Table 2 Levels of cytokines at day 3 (66 hours post infection) with no significant differences (Ns) between treated and untreated wounds (one-way ANOVA with Dunnett's post-hoc analysis).

Cytokine	Uninfected	No treatment	Fusidic acid		Mupirocin	
	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Significance (no treatment vs. treatment)	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Significance (no treatment vs. treatment)
IFN-γ	6.4	21.3	20.4	Ns	19.3	Ns
IL-4	1.1	4.6	4.5	Ns	3.9	Ns
IL-12p70	3.3	11.6	11.0	Ns	10.1	Ns
MDC	43.8	127.6	135.6	Ns	125.6	Ns

Table 3 Cytokine levels at day 3 (66 hours post infection) above measurable range (dynamic range).

Cytokine	Dynamic range	Concentration of cytokine in supernatant (Geometric mean, pg/mL)			
		Uninfected	No treatment	Fusidic acid	Mupirocin
IL-6	0.12 – 976	1560.0	2331.6	2322.0	2318.0
IL-8	0.08 – 750	4549.0	5420.2	5402.1	5394.9
IL-8 (HA)[3]	382.4 – 173600	6913.0[1]	175294	138870[1,2]	108896[1]
MCP-1	0.36 – 1500	5123.0	5767.9	5766.5	5757.0

[1] Cytokine level is within dynamic range.
[2] Cytokine level is partly within dynamic range (some measured datapoints outside range, but Geometric Mean inside range).
[3] IL-8 detection antibody which is used when high IL-8 levels are anticipated.

Table 4 Cytokine levels at day 3 (66 hours post infection) below measurable range (dynamic range).

Cytokine	Dynamic range	Concentration of cytokine in supernatant (Geometric mean, pg/mL)			
		Uninfected	No treatment	Fusidic acid	Mupirocin
IL-5	0.44 – 1124	0.01	0.06	0.01[1]	0.02[1]
IL-12/IL-23p40	0.78 – 4500	0	0.46	0[1]	0.10
IL-16	5.66 – 3750	0.66	0.90	0.57[1]	0.77[1]
IL-17A	1.48 – 7306	0.04	0.23	0.11	0.16
TNF-β	0.1 – 916	0.01	0.06[1]	0.04[1]	0.03[1]

[1] One or more of the samples were measured to contain 0 pg/mL of the cytokine and the geometric mean can then not be calculated , instead is the mean listed.

Bacterial load for different Staphylococcal strains in an *in vitro* skin model

The *in vitro* skin wound model was used to investigate infections with MRSA and two other staphylococcus strains to compare infection levels, differences in histology, and cytokines. In the experiment, two human clinical *S. aureus* isolates, 43484 (CA-MRSA PVL+) and 114864 (MSSA t008 PVL-) were used as well as a canine isolate, *S. pseudintermedius* DK729 (MRSP).

Each wound were infected with 1-4·10^6 CFU. After two days of infection the average number of bacteria increased to 1.5·10^9 CFU for *S. aureus* (CA-MRSA) 43484, 9.8·10^8 CFU for *S. aureus* (MSSA PVL-) 114864 and 4.9·10^8 CFU for *S. pseudintermedius* DK729 (Figure 4A). At day 4 post infection the number of bacteria decreased slightly to 1.4·10^9 CFU for CA-MRSA and to 6.5·10^8 CFU for MSSA PVL- (Figure 4B), thus the bacterial load in the wounds peaks around day 2. The bacterial load of *S. pseudintermedius* was only investigated on day 2.

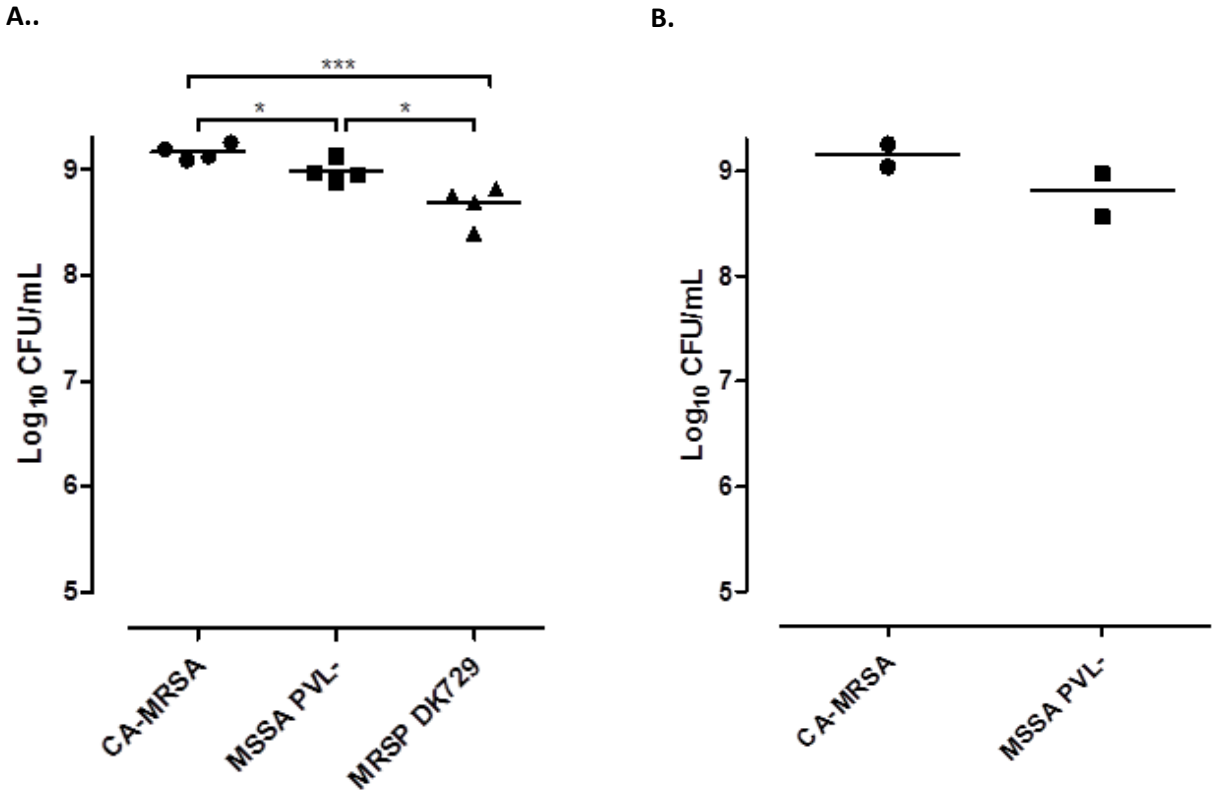


Figure 4 *In vitro* wounds in artificial skin infected with *S. aureus* (CA-MRSA t008 PVL+) 43484, *S. aureus* (MSSA t008 PVL-) 114864 and *S. pseudintermedius* (MRSP DK729). The wounds were infected with 1-4·10^6 CFU. The amount of bacteria per *in vitro* skin piece was determined at day 2 and 4 post-infection. **A.** Day 2; CFU per infected *in vitro* skin piece. **B**. Day 4; CFU per infected *in vitro* skin piece. The bar shows the geometric mean. Statistically differences between the three bacterial groups; * P <0.05 and *** P <0.001 (A).

PVL (Panton-Valentine leukocidin) is a cytotoxin that causes tissue destruction via lysis of the host's cells, and is found in some strains (isolates) of *S. aureus*. The presence of PVL is associated with increased virulence. Thus we expected the PVL negative (PVL-) isolate, 114864, to be less tissue-degrading than the 43484 PVL+ isolate. The two isolates are not isogenic, and they also differ with regard to Methicillin Resistance status, but they do belong to the same *spa* subtype (t008). *S. aureus* CA-MRSA reach the highest bacterial load of the three strains; however, inoculum for this strain was also the highest. The inoculum for the infection is based on measurement of absorbance (OD_{600}) assuming that at OD_{600} = 1 the bacterial concentration is 1·10^9 CFU/mL. Then plating is performed to get the actual CFU/ml, but this number is first available the following day, when colonies on the plates are counted. The differences in bacterial inocula mean that further trials are necessary to determine whether the infection capacity of the three bacterial strains truly differs.

Results from an experiment using the murine skin wound infection model developed by Lundberg and Frimodt-Møller, 2013[3] indicated that the *S. pseudintermedius* strain gave a lower bacterial load than MRSA, so we also expected to see something similar in the experiments using the in vitro skin wound infection model. *S. pseudintermedius* is often found as part of the common flora in domestic animals (especially in dogs) and it is an opportunistic pathogen that can infect any tissue, but it usually causes skin infections. *S. pseudintermedius*, like *S. aureus*, can be both methicillin-susceptible (MSSP) and methicillin-resistant (MRSP), and the latter phenotype is a major health problem in dogs, as MRSA is a major problem in humans. It is rare that *S. pseudintermedius* infections have been registered in humans, which may be due to the fact that *S. pseudintermedius* can easily be confused with *S. aureus*.

In an earlier study conducted at Statens Serum Institut (SSI), using the murine skin wound infection model developed by Lundsberg and Frimodt-Møller, the *S. pseudintermedius* DK729 (also used here), was shown to colonise only at low levels at day 4 (average around $1\text{-}5\cdot10^6$ CFU/g but with a high level of variation between data points, unpublished data, Peter Damborg). Infection with *S. aureus* strains in the murine model normally results in a bacterial load around $1\cdot10^8$ CFU/g at day 4. Neither human nor mouse is *S. pseudintermedius*' common host, and it is likely that it adheres better to the tissue of dogs (the primary host of *S. pseudintermedius*) than other species. In our experiments, it was also noted that *S. pseudintermedius* were easily washed off the skin during harvesting. *S. pseudintermedius* DK729 were also found to result in the lowest bacterial load (at day 2) of the three strains, however, inoculum for this strain was also the lowest among the three strains investigated.

Figure 5 shows histology sections of four *in vitro* wounded skin pieces 2 days post infection with one of three staphylococci strains or left uninfected. The histology shows that a large amount of bacteria (seen as dark purple areas in the histology images) are found at the wound periphery (Figure 5 BCD), adhering to the crack left by the biopsy needle. Debris from broken keratinocytes and fibroblasts may also have made this area more favourable to the bacteria.

The uninfected wounds showed clear signs of partial healing already after 2 days, with the keratinocytes migrating towards the centre of the wound (Figure 5 A). Bacteria in high numbers had penetrated deeply into dermis in the skin pieces with wounds infected with MRSA and MSSA (Figure 5 B&C, respectively). In addition, the bacteria apparently also caused the epidermis to detach from the dermis, perhaps by secreting virulence factors such as proteases killing cells in the *in vitro* skin.
The MRSP strain (Figure 5 D) may be less virulent than MRSA and MSSA; few bacteria of this strain has penetrated deeply into the dermis, moreover, the epidermis is still attached to the dermis although detachment can be seen near the wound edges.

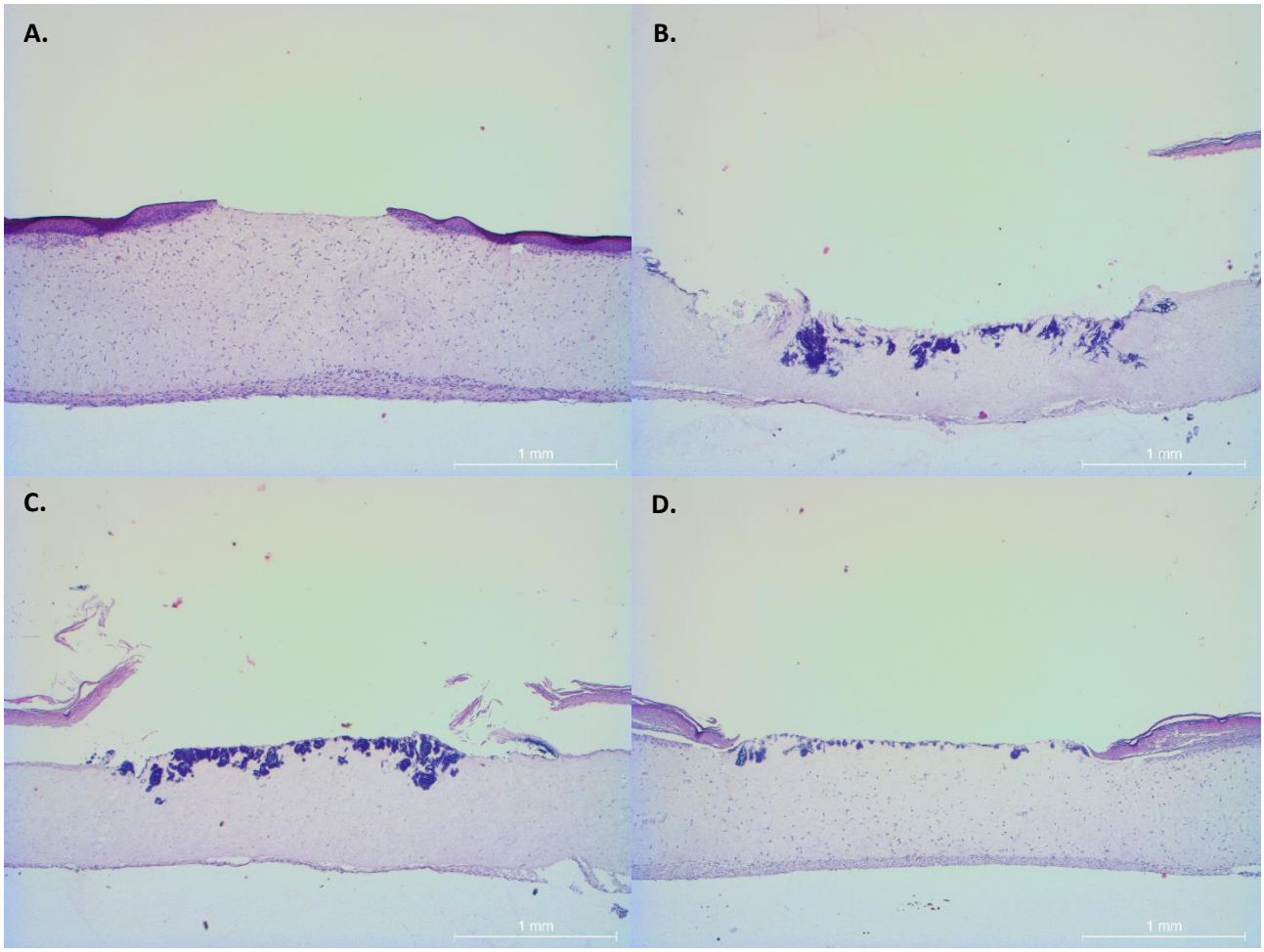


Figure 5 Histology sections of the *in vitro* wound 2 days (48 hours) post infection with one of three different staphylococci strains. Bacteria appear as dark purple areas. **A.** Uninfected wound. **B.** Wound infected with *S. aureus* (CA-MRSA PVL+) 43484. C. Wound infected with *S. aureus* (MSSA PVL-). **D.** Wound infected with *S. pseudintermedius* (MRSP) DK729.

The concentration of 29 cytokines and chemokines in the supernatants from the wounded *in vitro* skin pieces (either uninfected or infected with one of the three staphylococci strains) were measured, and of these were 17 within (Table 5 and Table 6), 7 above (Table 7) and 5 below (Table 8) the measurable range.

In the supernatant from the wounded and infected *in vitro* skin pieces the concentration increased for 27 of the cytokines and chemokines compared to the supernatant for wounded and uninfected skin pieces, and 14 of these (which were within the measurable range) increased significantly (Table 5). The concentration of IL-10, an anti-inflammatory cytokine, was significantly higher in the supernatant from wounded skin pieces infected with the MRSP strain compared to supernatant from wounded skin pieces infected with either MRSA or MSSA (Table 9). This increase agrees with the significant decrease in the pro-inflammatory cytokines TNF, IL-8, GM-CFS and IL-1β comparing supernatants from MRSP infected skin pieces to skin pieces infected with either MRSA or MSSA. The synthesis of these four latter cytokines is inhibited by IL-10.
Comparing the cytokine profile in the supernatant from the PVL+ MRSA strain with the PVL- MSSA only reveals few differences; INF-γ and IL-2 is higher in the supernatant from wounded skin pieces infected with the PVL- MSSA strain than in the supernatant from wounded skin pieces infected with the PVL+ MRSA, whereas for IL-15 the image is reversed.

Table 5 Levels of cytokines at day 2 (48 hours post infection) with significant differences between uninfected and infected wounds. Statistically significant difference: Ns (non-significant), * (P<0.05), ** (P<0.01) and *** (P<0.001). ↑/↓ Infected significantly higher/lower than uninfected. (One-way ANOVA with Dunnett's post-hoc analysis).

Cytokine	Uninfected	CA-MRSA PVL+		MSSA PVL-		MRSP DK729	
	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Significance (uninfected vs. infected)	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Significance (uninfected vs. infected)	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Significance (uninfected vs. infected)
Eotaxin-3	9.6	45.6	*↑	51.9	**↑	35.5	Ns
IFN-γ	1.9	22.3	***↑	26.5	***↑	26.9	***↑
IL-1β	0.5	17.7	***↑	19.4	***↑	7.1	**↑
IL-2	0.4	8.0	***↑	9.8	***↑	8.8	***↑
IL-4	0.7	5.3	**↑	7.1	***↑	6.5	***↑
IL-10	0.9	4.4	***↑	5.3	***↑	8.5	***↑
IL-12p70	1.9	12.5	**↑	15.5	***↑	12.1	**↑
IL-13	6.4	27.1	***↑	31.5	***↑	31.1	***↑
IL-15	1.5	10.3	***↑	8.1	***↑	3.6	***↑
MDC	16.2	102.9	***↑	116.7	***↑	95.7	***↑
TARC	1.2	8.5	*↑	11.0	**↑	8.5	*↑
TNF-α	1.9	17.5	***↑	18.9	***↑	13.9	***↑
IP-10	50.6	6.9	***↓	16.2	**↓	21.0	**↓
IL-7	3.6	1.8	**↓	2.0	**↓	4.0	Ns

Table 6 Cytokine levels at day 2 (48 hours post infection) with no significant differences (Ns) between uninfected and infected wounds.

Cytokine	Uninfected	CA-MRSA PVL+		MSSA PVL-		MRSP DK729	
	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Significance (uninfected vs. infected)	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Significance (uninfected vs. infected)	Concentration of cytokine in supernatant (Geometric mean, pg/mL)	Significance (uninfected vs. infected)
Eotaxin-1	27.9	56.7	Ns	333.0	Ns	116.7	Ns
IL-16	13.1	68.0[1]	Ns	64.2	Ns	192.9	Ns
TNF-β	1.1	2.6	Ns	3.2	Ns	1.7[1]	Ns

[1] One or more of the samples were measured to contain 0 pg/mL of the cytokine and the geometric mean can then not be calculated , instead is the mean listed.

Table 7 Cytokine levels at day 2 (48 hours post infection) above measurable range (dynamic range).

Cytokine	Dynamic range	Concentration of cytokine in supernatant (Geometric mean, pg/mL)			
		Uninfected	**CA-MRSA PVL+**	**MSSA PVL-**	**MRSP DK729**
IL-1α	0.18-556	4.9[1]	1747.2	1534.4	41.61
IL-6	0.12 – 976	1566.2	2331.9	2357.5	2377.8
IL-8	0.08 – 750	3069.0	5552.0	5616.6	5443.1
IL-8 (HA)[2]	382.4 – 173600	2807.5[1]	196,226	241,339	79,662.4[1]
MCP-1	0.36 – 1500	1639.4	5719.5	5724.8	5579.3
GM-CFS	0.28-1500	13.7[1]	874.1[1]	1779.4	241.4[1]
VEGF-A	2.24-1568	1197.1[1]	1591.1	1353.2[1]	2958.5

[1] Cytokine level are within dynamic range.

[2] IL-8 detection antibody which is used when high IL-8 levels are anticipated.

Table 8. Cytokine levels at day 2 (48 hours post infection) below measurable range (dynamic range).

Cytokine	Dynamic range	Concentration of cytokine in supernatant (Geometric mean, pg/mL)			
		Uninfected	**CA-MRSA PVL+**	**MSSA PVL-**	**MRSP DK729**
IL-5	0.44 – 1124	0.2	0.7[2]	0.7[2]	0.3[1]
IL-12/IL-23p40	0.78 – 4500	0.3	2.9[2]	3.4[2]	3.2[2]
IL-17A	1.48 – 7306	0.01	0.04	0.06	0.06
MIP-1α	12.08-2972	7.5	26.8[2]	38.1[2]	35.3[2]
MIP-1β	1.48-3000	0.6	4.9[2]	5.7[2]	5.0[2]

[1] One or more of the samples were measured to contain 0 pg/mL of the cytokine and the geometric mean can then not be calculated , instead is the mean listed.
[2] Cytokine level is within dynamic range.

Table 9 Significant differences in the cytokine levels at day 2 (48 hours post infection) between the infected *in vitro* wounds. Statistically significant difference: Ns (no significance), * (P<0.05), ** (P<0.01) and *** (P<0.001). ↑/↓ Significantly higher/lower. E.g. the cytokine level for IL-7 was significantly higher (***↑) in MRSP compared to MRSA.

Cytokine	Significance		
	CA-MRSA PVL+ vs. MSSA PVL-	CA-MRSA PVL+ vs. MRSP DK729	MSSA PVL- vs. MRSP DK729
IL-7	Ns	***↑	***↑
IL-10	Ns	***↑	***↑
IL-1β	Ns	***↓	***↓
IP-10	Ns	**↑	Ns
IFN-γ	*↑	*↑	Ns
IL-2	*↑	Ns	Ns
IL-15	***↓	***↓	***↓

Conclusion

The aim of this project has been to develop, implement and evaluate an *in vitro* skin infection model to use as an alternative to animal models for test of the efficacy of topical antimicrobial compounds and investigation of bacterial load by different pathogens.

The results achieved in our experiment using the *in vitro* model for studying the efficacy of two topical antimicrobials (fusidic acid and mupirocin) corresponded well to the results obtained by Lundberg and Frimodt-Møller using the murine *in vivo* wound infection model. Both models show a significant reduction in bacterial load after treatment with the two antimicrobials, moreover, both model shows that mupirocin has a higher antimicrobial efficacy than fusidic acid.

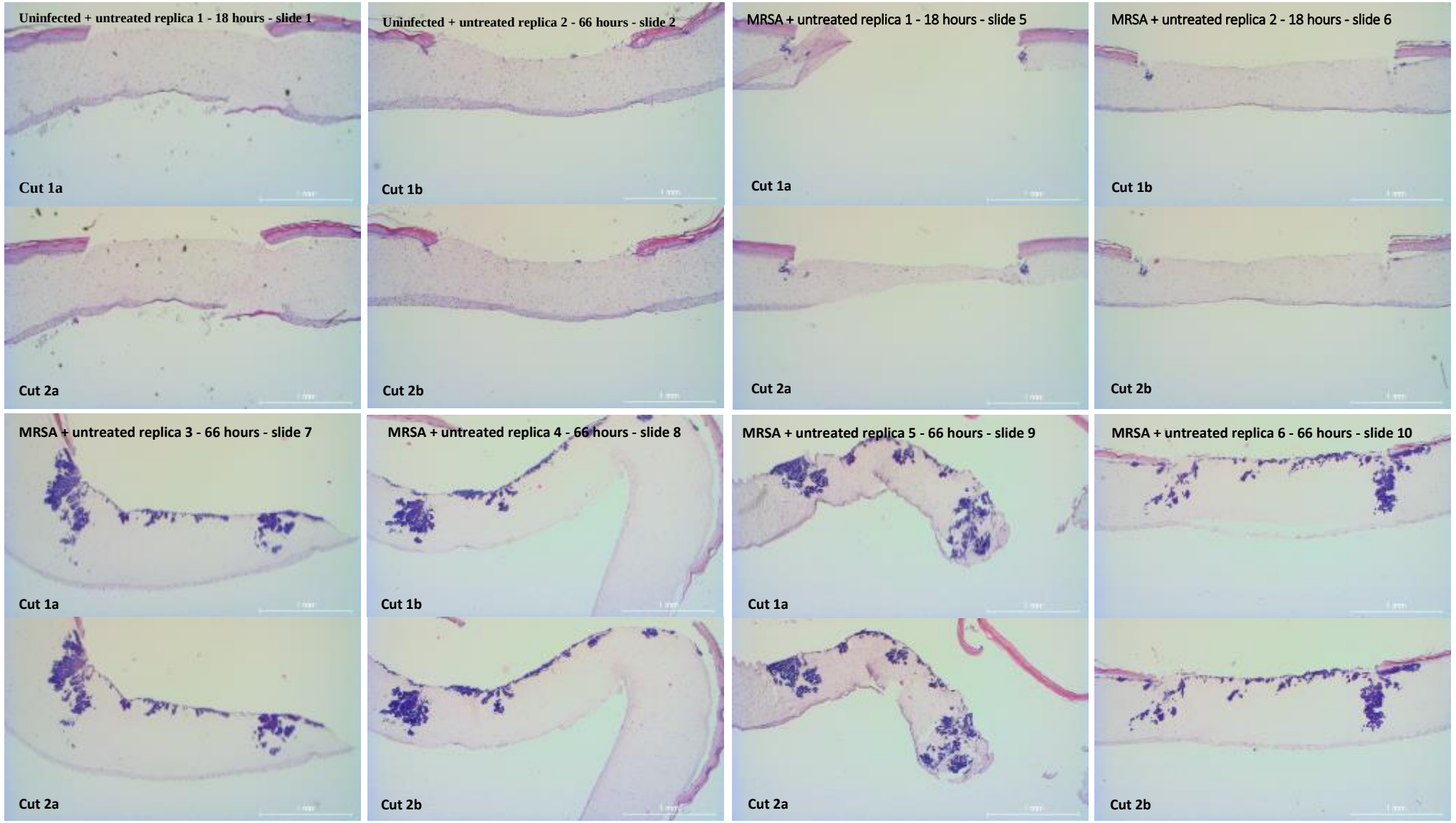
As we have shown here, the model not only allows measurement of the bacterial load, but also enables investigation of wound healing by histology. The histology also makes is possible to follow localisation of the bacteria e.g. revealing that the bacteria may penetrate deeply into the dermis.
By measurement of cytokines in the supernatant (spent media) of the *in vitro* skin pieces, we could follow changes in the cytokine and chemokine profile in response to infection and treatment. Not surprisingly, we found that infection induced a strong inflammatory response; however, we also showed that after two days of antimicrobial treatment, the cytokine profile moved significantly away from the proinflammatory response towards the profile of the uninfected samples.

We also used the model to investigate three different strains of the genus Staphylococcus, and found that the canine isolate *S. pseudintermedius* strain, MRSP DK729 was less virulent than two human *S. aureus* isolates, with MRSP DK729 penetrating less into dermis and causing a lower inflammatory response.
Interestingly, the PVL- and the PVL+ *S. aureus* isolates behaved very similar in the model. PVL (Panton-Valentine leucocidin) is a toxin associated with increased virulence; however, the presence of the toxin had apparently only a minor effect on the cytokine profile and no effect on the bacterial load.
Thus to summarize the above, we have shown that the *in vitro* wound infection model we have developed can be used for examining the bacterial load before and after topical treatment, and can be used to compare bacterial load of different strains of bacteria. Moreover, the model can be used for investigation of infections based on histology and cytokine profile. Thus the model offers a strong alternative to animal models for research in wound infections, including but not limited to investigations of the efficacy of topical antimicrobial compounds.

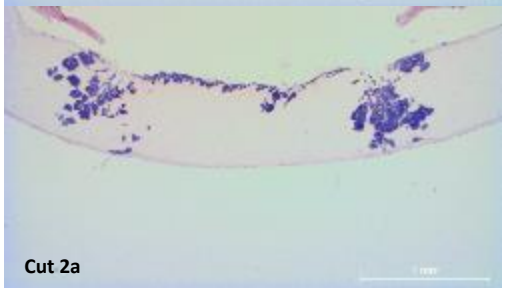
References

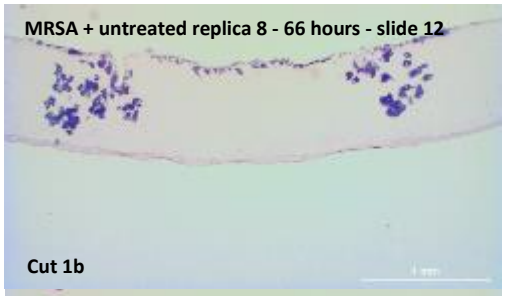
1. Hvor mange forsøgsdyr bruges i Danmark @ 3rcenter.dk. Available at: http://3rcenter.dk/forsoegsdyr/hvor-mange-forsoegsdyr-bruges.
2. Sygdomsdata @ www.ssi.dk. Available at: http://www.ssi.dk/Smitteberedskab/Sygdomsovervaagning/Sygdomsdata.aspx?sygdomskode=MRSA&xaxis=Aar&show=&datatype=Laboratory&extendedfilters=False#HeaderText.
3. Lundberg, C. V. & Frimodt-Møller, N. International Journal of Antimicrobial Agents Efficacy of topical and systemic antibiotic treatment of meticillin-resistant Staphylococcus aureus in a murine superficial skin wound infection model. *Int. J. Antimicrob. Agents* **42,** 272–275 (2013).
4. Shankar Subramanian Iyer and Genhong Cheng. Role of Interleukin 10 Transcriptional Regulation in Inflammation and Autoimmune Disease. *Crit. Rev. Immunol.* **32,** 23–63 (2012).
5. Duque, G. A. & Descoteaux, A. Macrophage cytokines : involvement in immunity and infectious diseases. *Front. Immunol.* **5,** 1–12 (2014).
6. Graham, G. J. and Locati, M. Regulation of the immune and inflammatory responses by the 'atypical' chemokine receptor D6. *J. Pathol.* **229,** 168–175 (2013).

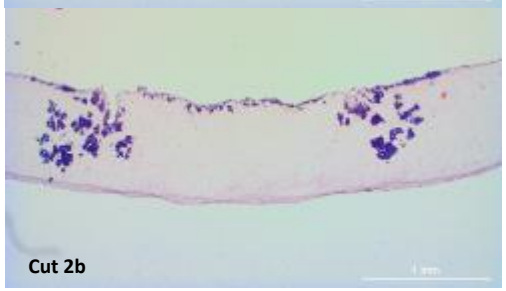
Appendix A - Topical treatment of methicillin-resistant *Staphylococcus aureus* in an *in vitro* skin wound infection model – Histology

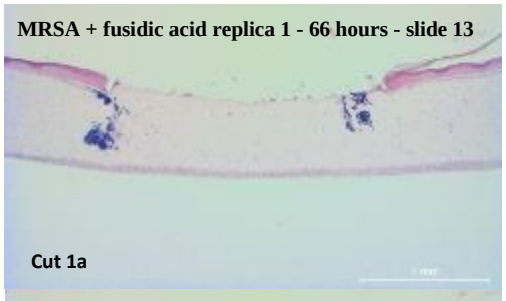


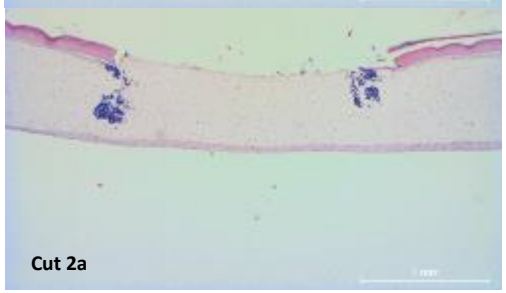

MRSA + untreated replica 7 - 66 hours - slide 11
Cut 1a

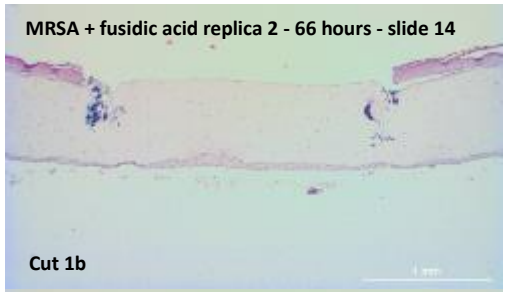

Cut 2a

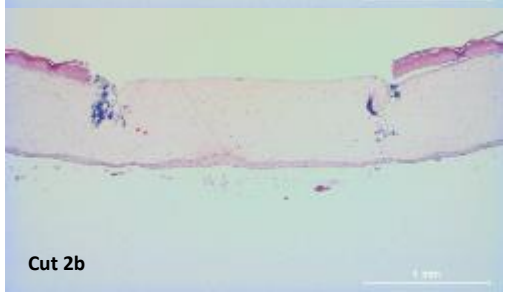

MRSA + untreated replica 8 - 66 hours - slide 12
Cut 1b

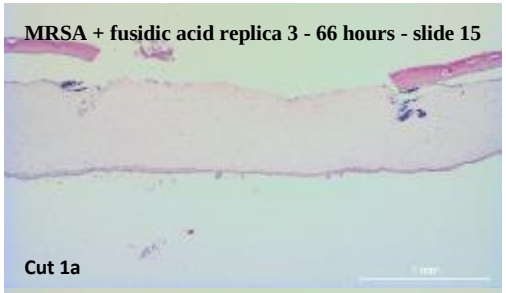

Cut 2b

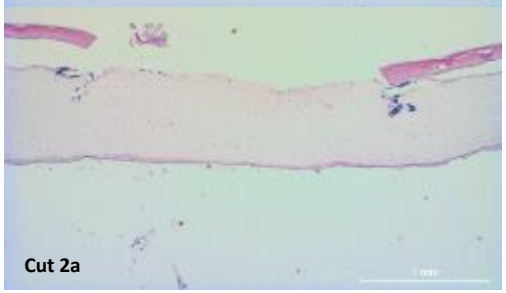

MRSA + fusidic acid replica 1 - 66 hours - slide 13
Cut 1a

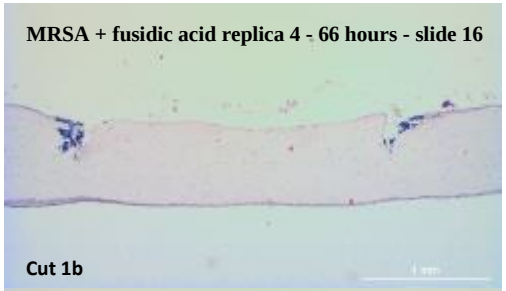

Cut 2a

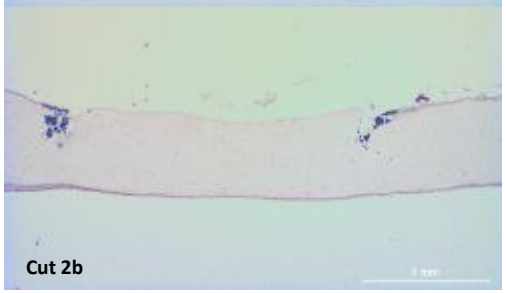

MRSA + fusidic acid replica 2 - 66 hours - slide 14
Cut 1b

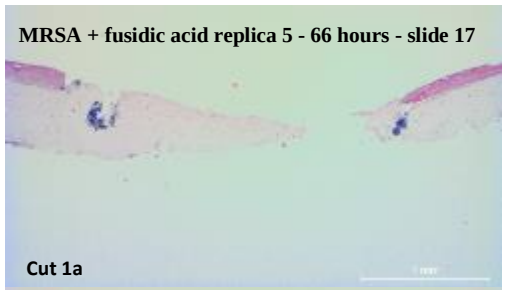

Cut 2b


MRSA + fusidic acid replica 3 - 66 hours - slide 15
Cut 1a

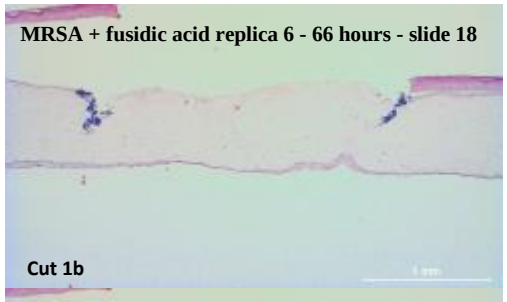

Cut 2a


MRSA + fusidic acid replica 4 - 66 hours - slide 16
Cut 1b


Cut 2b

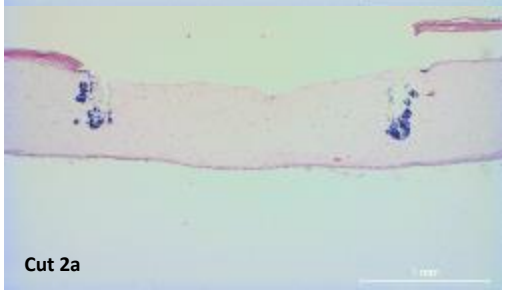

MRSA + fusidic acid replica 5 - 66 hours - slide 17
Cut 1a


Cut 2a

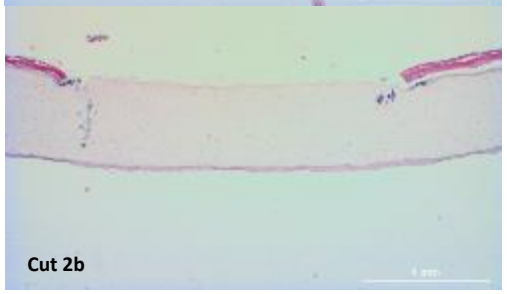

MRSA + fusidic acid replica 6 - 66 hours - slide 18
Cut 1b


Cut 2b

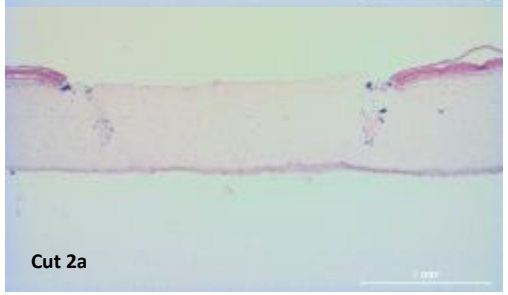

MRSA + mupirocin 1 - 66 hours - slide 19
Cut 1a


Cut 2a

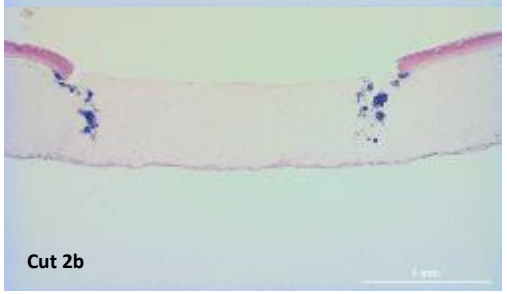

MRSA + mupirocin replica 2 - 66 hours - slide 20
Cut 1b

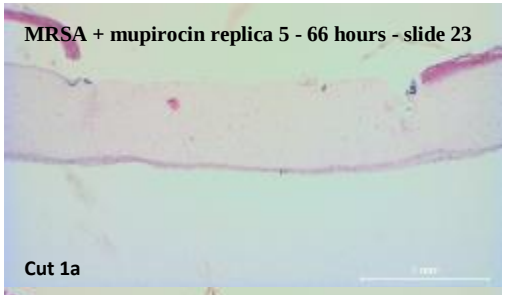

Cut 2b

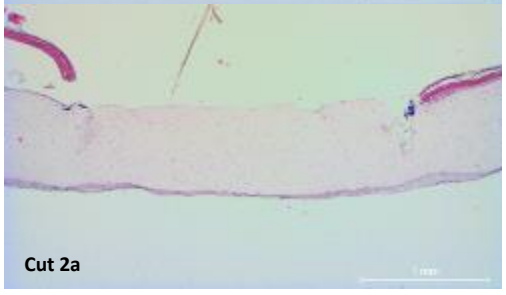

MRSA + mupirocin replica 3 - 66 hours - slide 21
Cut 1a

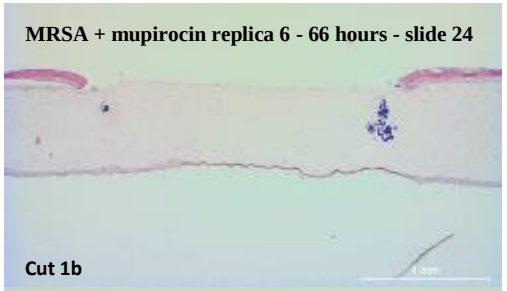

Cut 2a

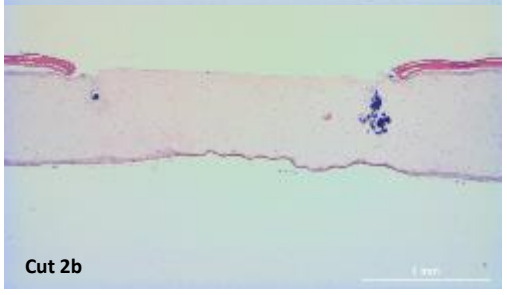

MRSA + mupirocin replica 4 - 66 hours - slide 22
Cut 1b


Cut 2b

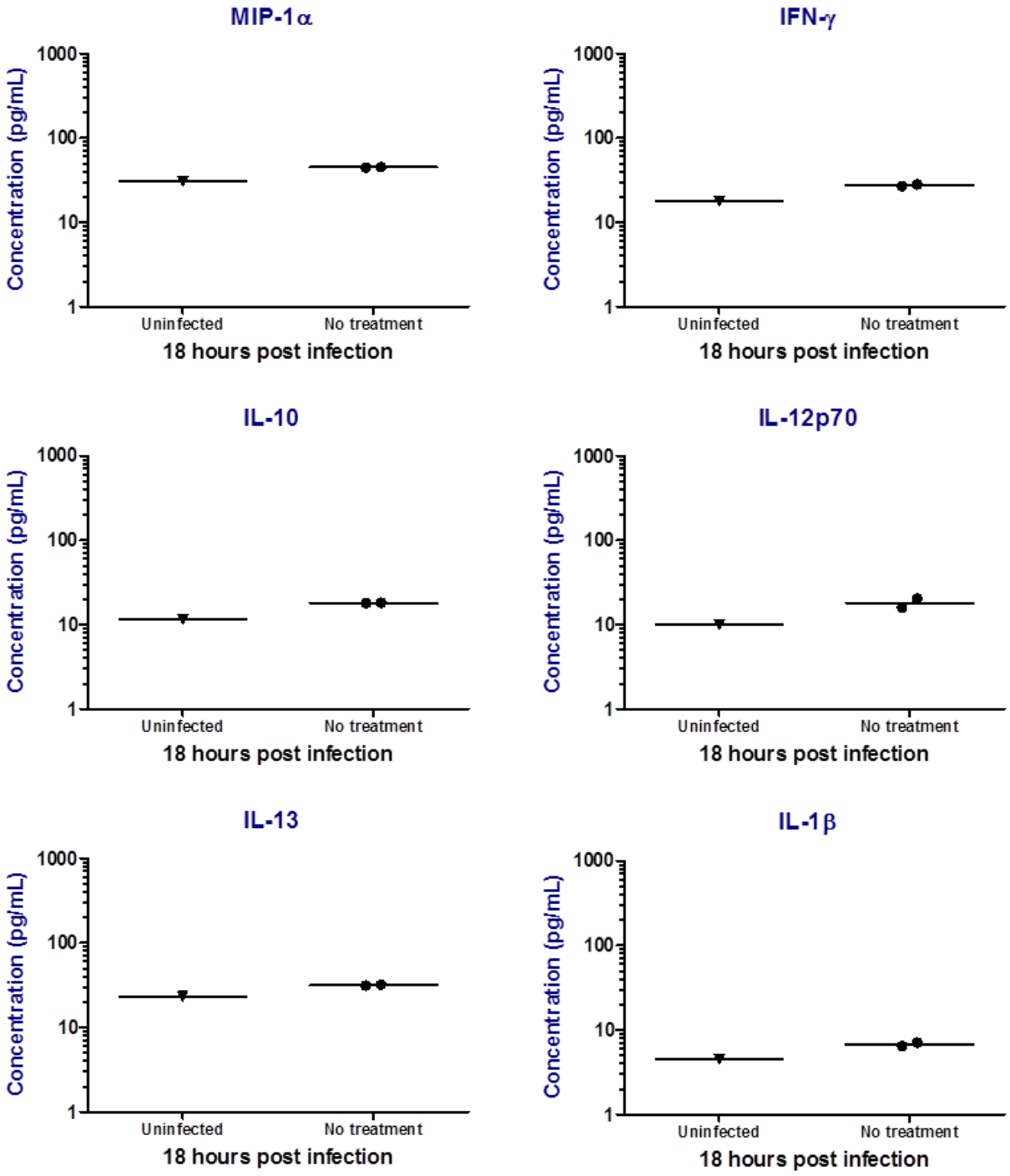

MRSA + mupirocin replica 5 - 66 hours - slide 23
Cut 1a

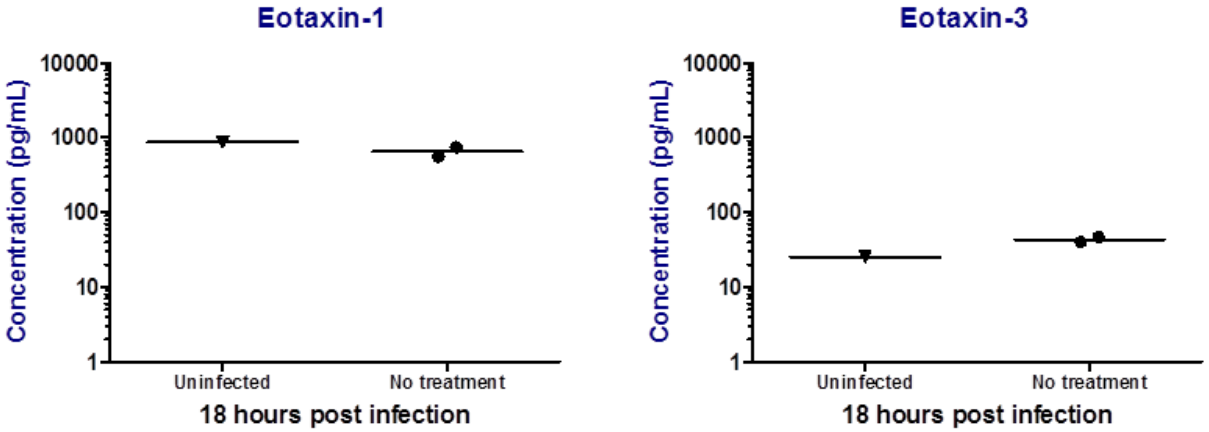

Cut 2a


MRSA + mupirocin replica 6 - 66 hours - slide 24
Cut 1b

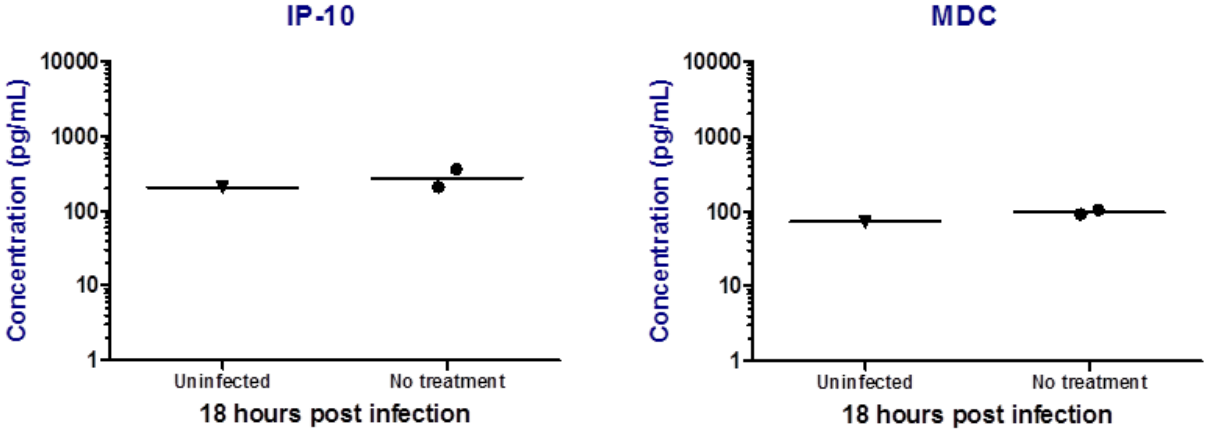

Cut 2b

Appendix B – Topical treatment of methicillin-resistant Staphylococcus aureus in an in vitro skin wound infection model – Cytokines and chemokines

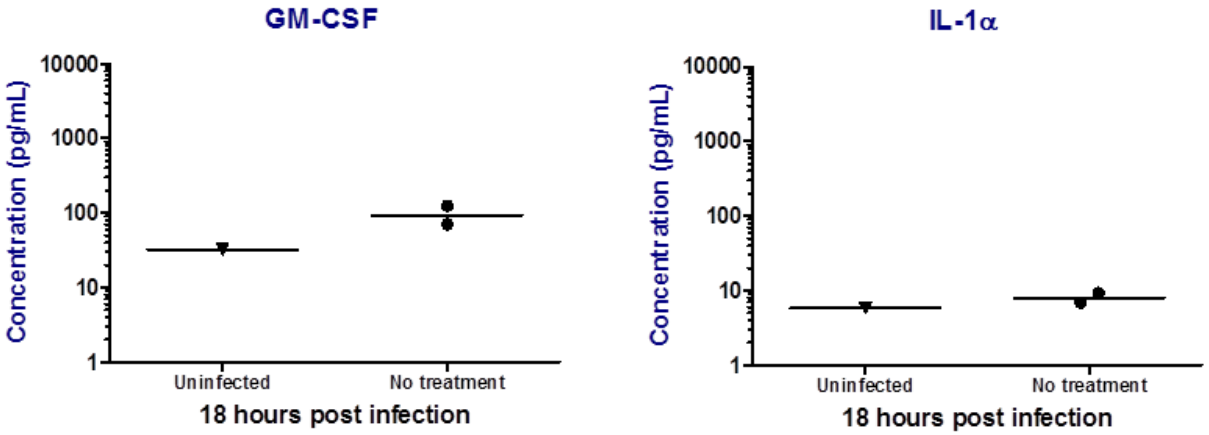



Eotaxin-1
Concentration (pg/mL)
10000
1000
100
10
1
Uninfected
No treatment
18 hours post infection

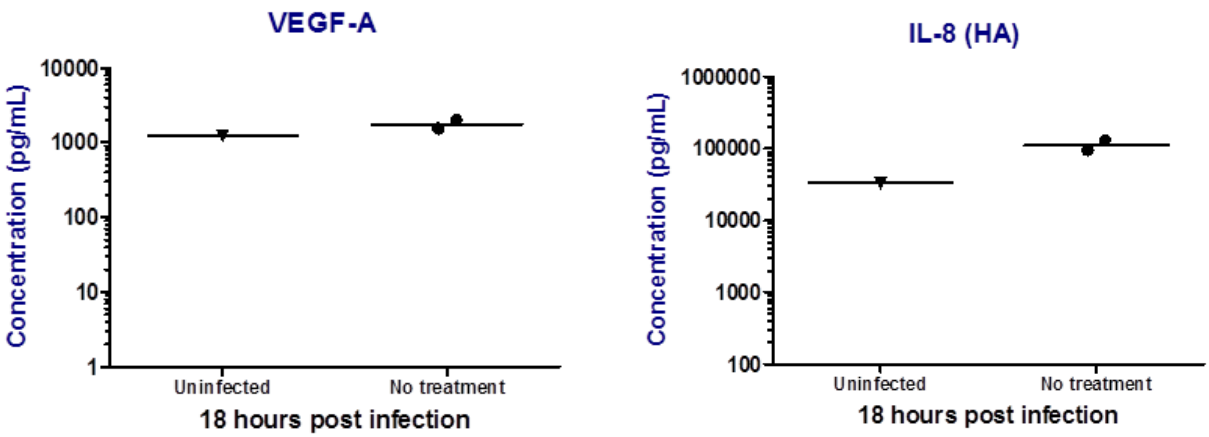
Eotaxin-3
Concentration (pg/mL)
10000
1000
100
10
1
Uninfected
No treatment
18 hours post infection


IP-10
Concentration (pg/mL)
10000
1000
100
10
1
Uninfected
No treatment
18 hours post infection

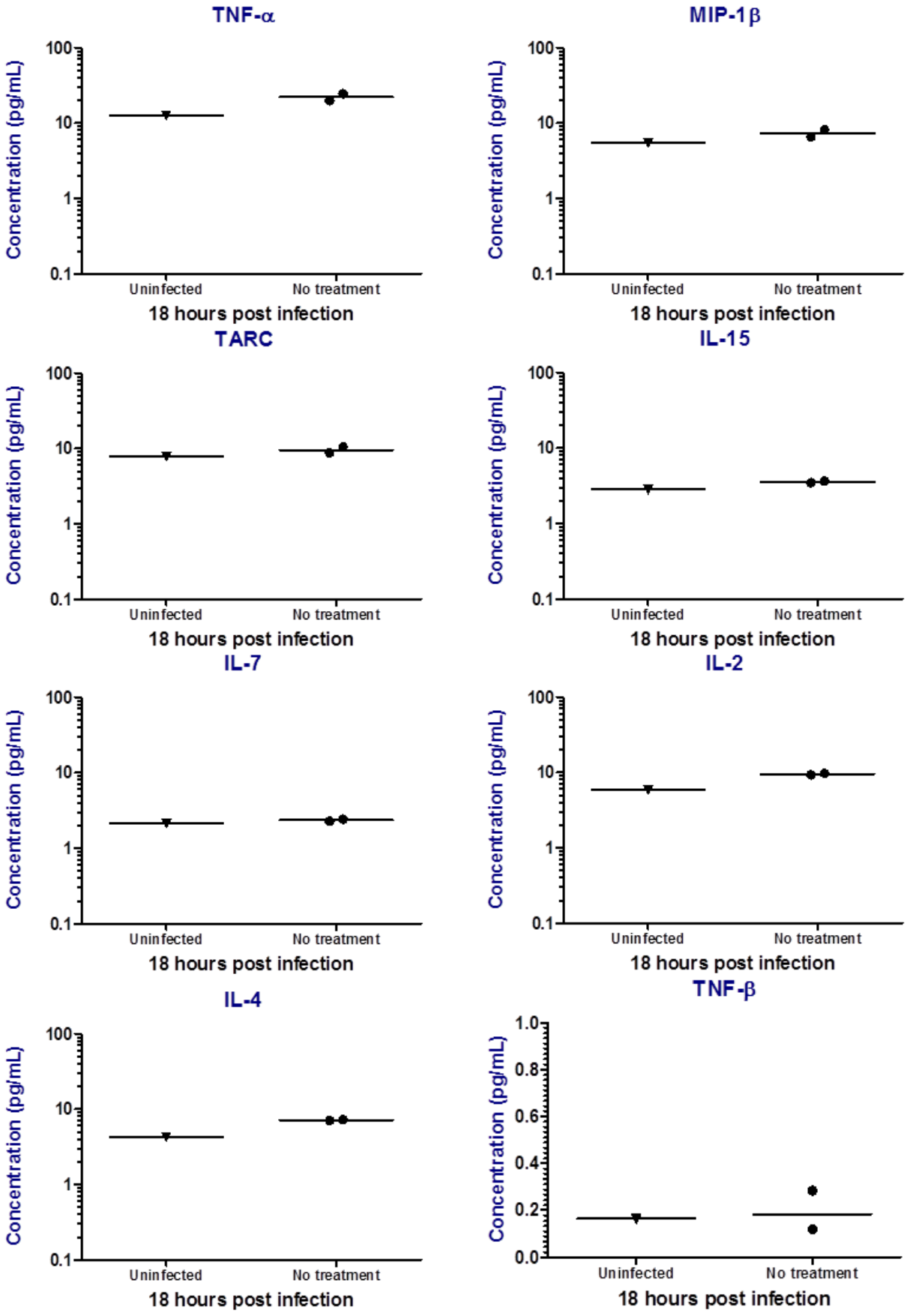
MDC
Concentration (pg/mL)
10000
1000
100
10
1
Uninfected
No treatment
18 hours post infection

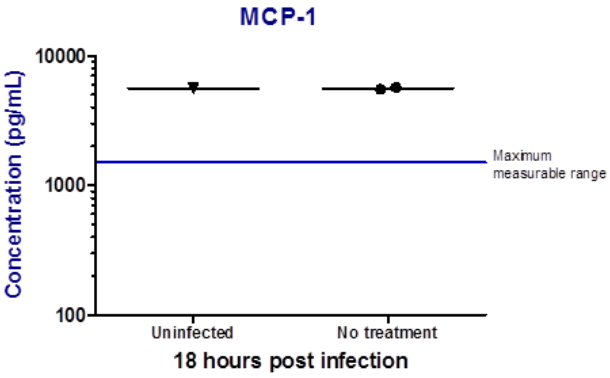

GM-CSF
Concentration (pg/mL)
10000
1000
100
10
1
Uninfected
No treatment
18 hours post infection

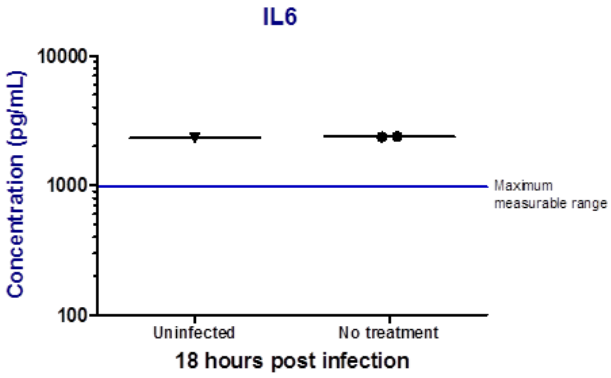
IL-1α
Concentration (pg/mL)
10000
1000
100
10
1
Uninfected
No treatment
18 hours post infection

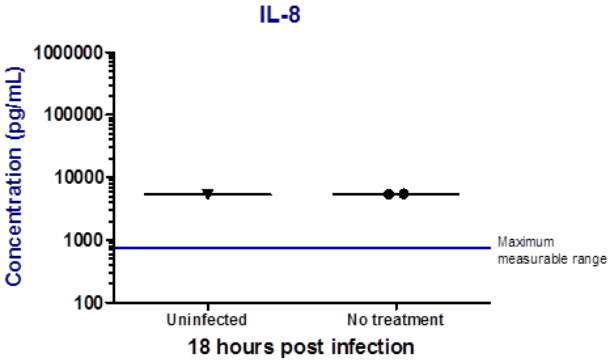

VEGF-A
Concentration (pg/mL)
10000
1000
100
10
1
Uninfected
No treatment
18 hours post infection

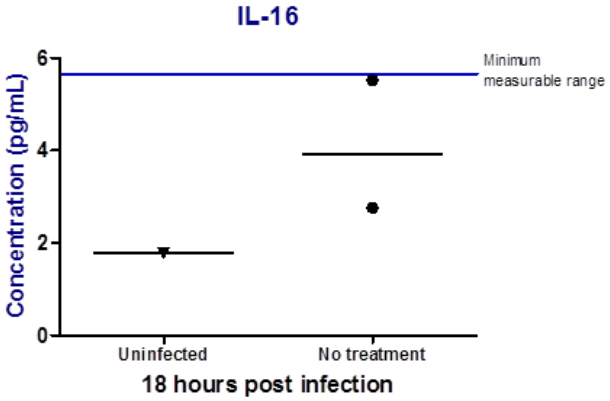
IL-8 (HA)
Concentration (pg/mL)
1000000
100000
10000
1000
100
Uninfected
No treatment
18 hours post infection

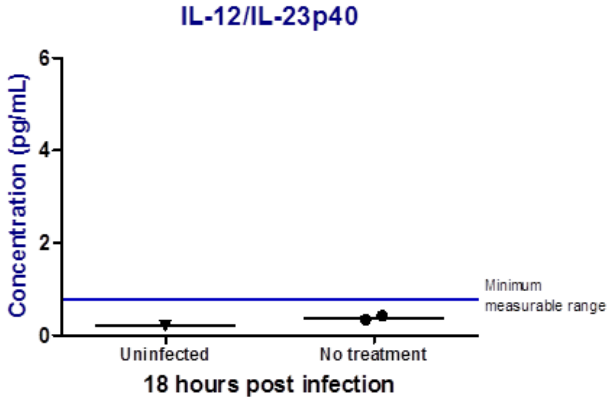

TNF-α
MIP-1β
TARC
IL-15
IL-7
IL-2
IL-4
TNF-β
Concentration (pg/mL)
100
10
1
0.1
1.0
0.8
0.6
0.4
0.2
0.0
Uninfected
No treatment
18 hours post infection

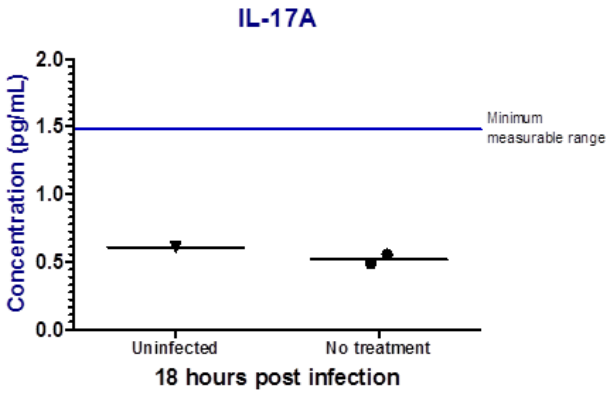

MCP-1
Concentration (pg/mL)
10000
1000
100
Maximum measurable range
Uninfected
No treatment
18 hours post infection

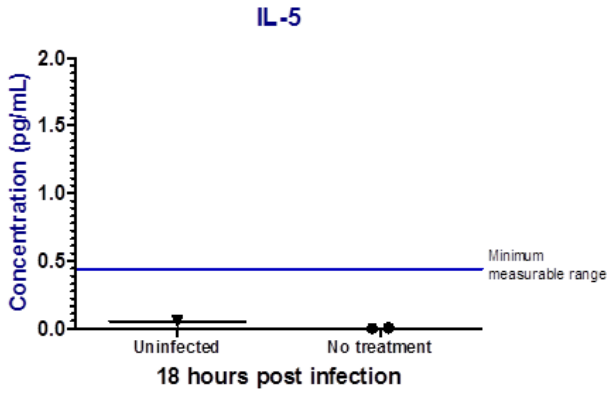

IL6
Concentration (pg/mL)
10000
1000
100
Maximum measurable range
Uninfected
No treatment
18 hours post infection

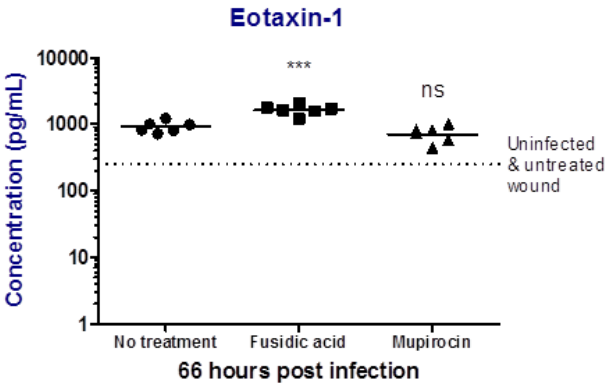

IL-8
Concentration (pg/mL)
1000000
100000
10000
1000
100
Maximum measurable range
Uninfected
No treatment
18 hours post infection

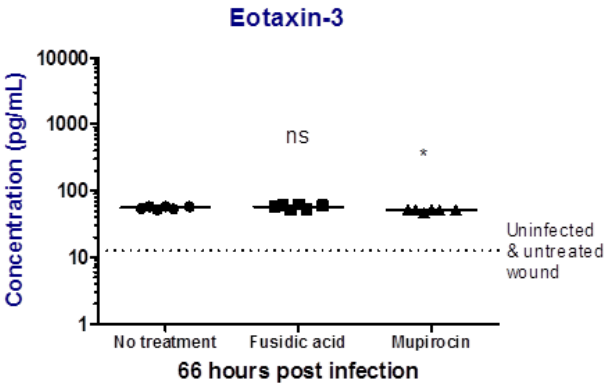

IL-16
Concentration (pg/mL)
6
4
2
0
Minimum measurable range
Uninfected
No treatment
18 hours post infection

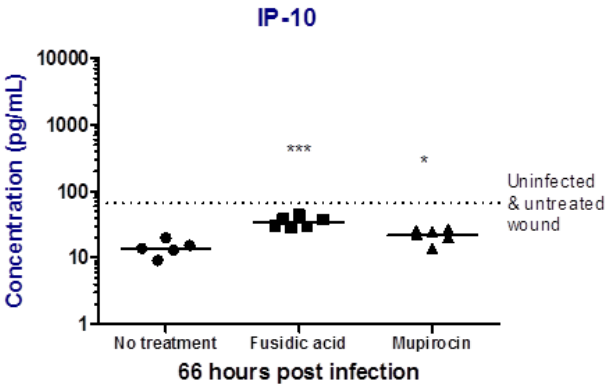

IL-12/IL-23p40
Concentration (pg/mL)
6
4
2
0
Minimum measurable range
Uninfected
No treatment
18 hours post infection

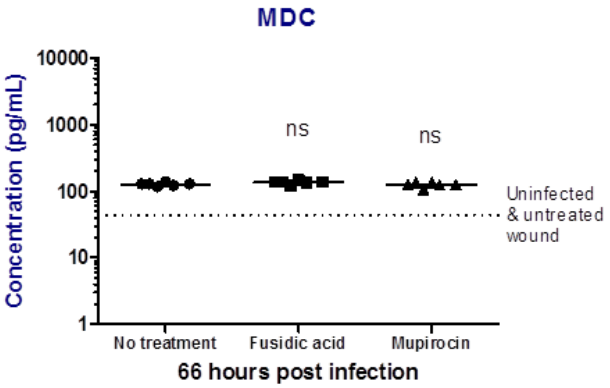

IL-17A
Concentration (pg/mL)
2.0
1.5
1.0
0.5
0.0
Minimum measurable range
Uninfected
No treatment
18 hours post infection

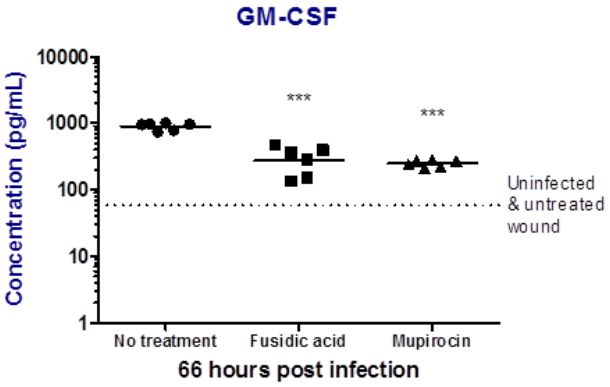

IL-5
Concentration (pg/mL)
2.0
1.5
1.0
0.5
0.0
Minimum measurable range
Uninfected
No treatment
18 hours post infection

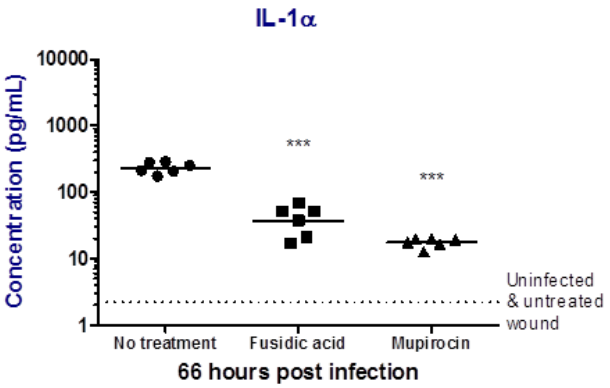

Eotaxin-1
Concentration (pg/mL)
10000
1000
100
10
1
***
ns
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

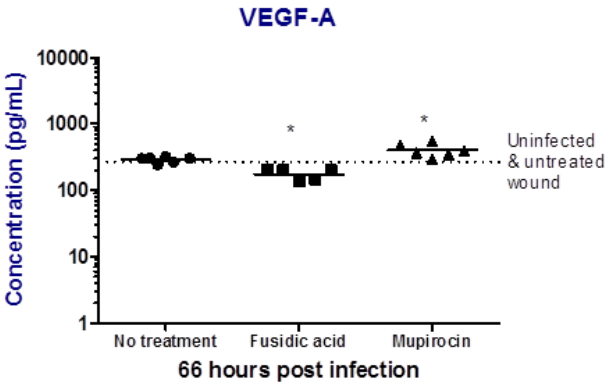

Eotaxin-3
Concentration (pg/mL)
10000
1000
100
10
1
ns
*
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

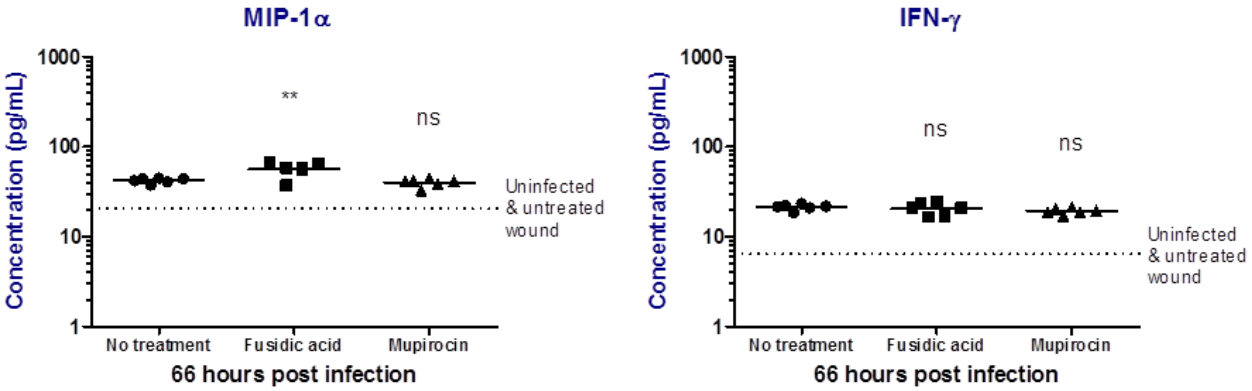

IP-10
Concentration (pg/mL)
10000
1000
100
10
1
***
*
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection


MDC
Concentration (pg/mL)
10000
1000
100
10
1
ns
ns
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

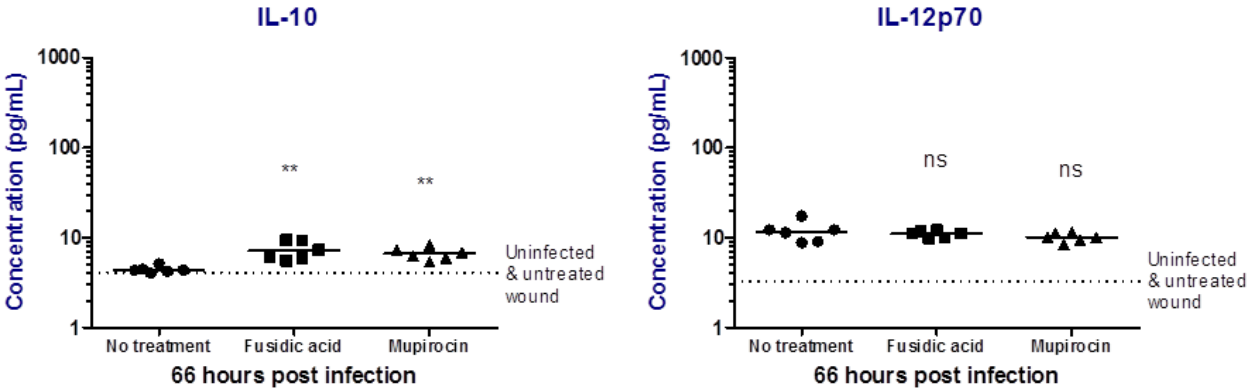

GM-CSF
Concentration (pg/mL)
10000
1000
100
10
1
***
***
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection


IL-1α
Concentration (pg/mL)
10000
1000
100
10
1
***
***
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

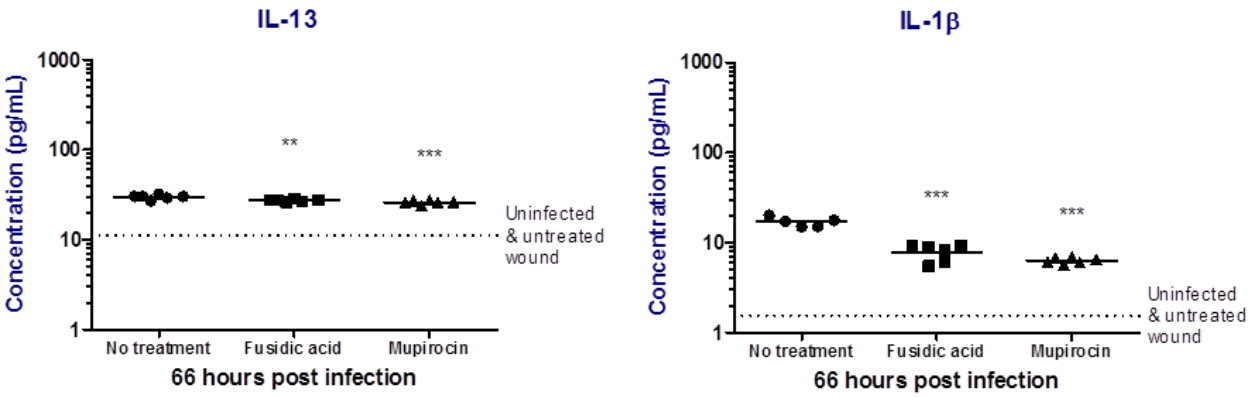

VEGF-A
Concentration (pg/mL)
10000
1000
100
10
1
*
*
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection


MIP-1α
Concentration (pg/mL)
1000
100
10
1
**
ns
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

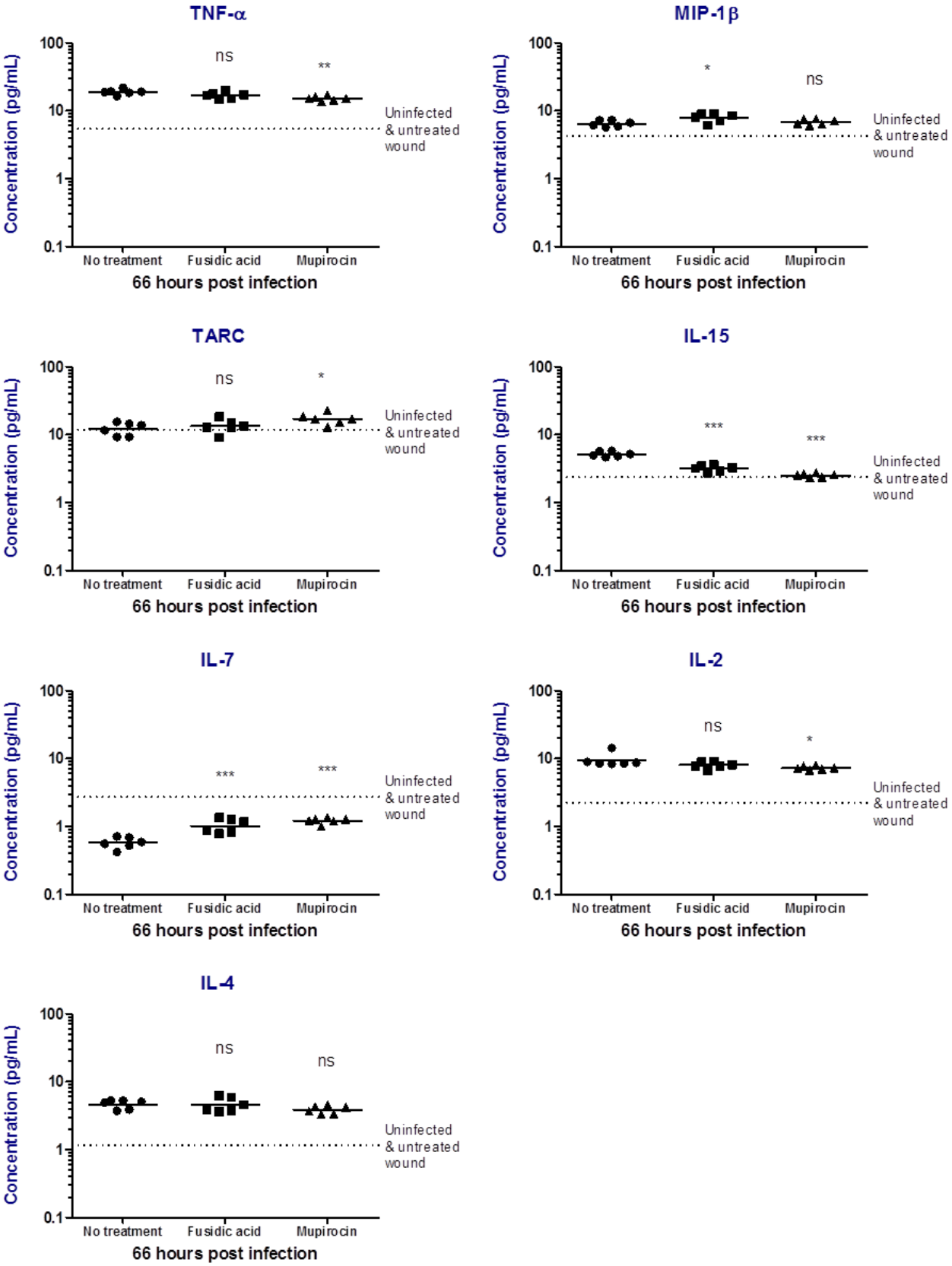
IFN-γ
Concentration (pg/mL)
1000
100
10
1
ns
ns
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

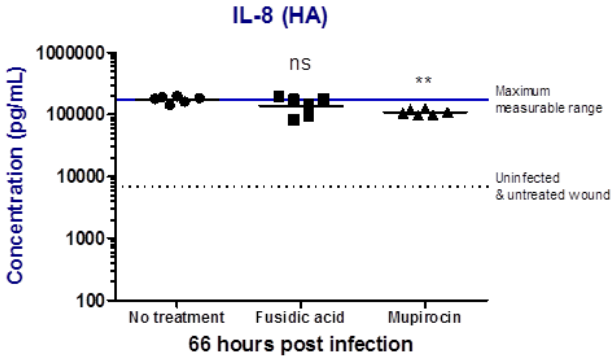

IL-10
Concentration (pg/mL)
1000
100
10
1
**
**
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

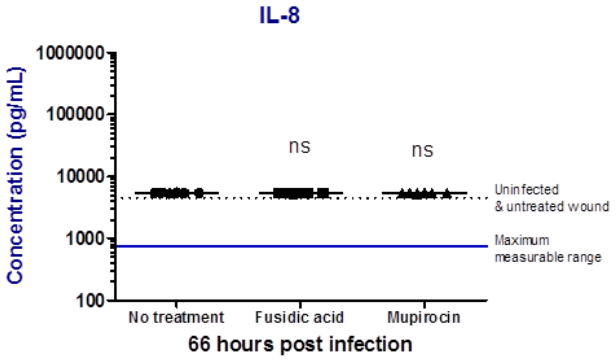
IL-12p70
Concentration (pg/mL)
1000
100
10
1
ns
ns
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

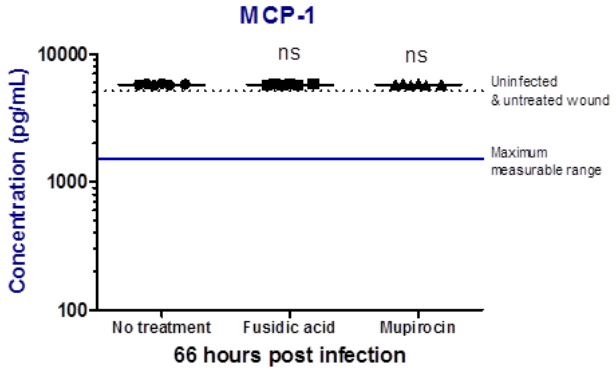

IL-13
Concentration (pg/mL)
1000
100
10
1
**
***
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

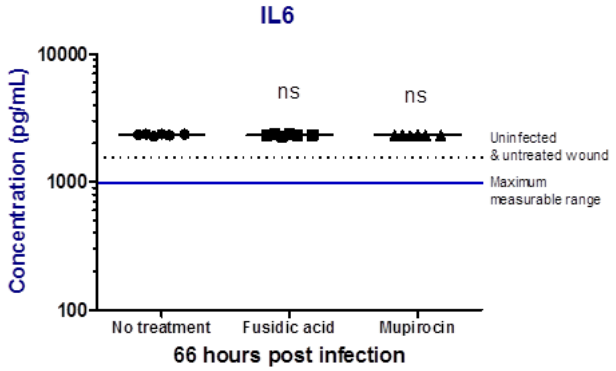
IL-1β
Concentration (pg/mL)
1000
100
10
1
***
***
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

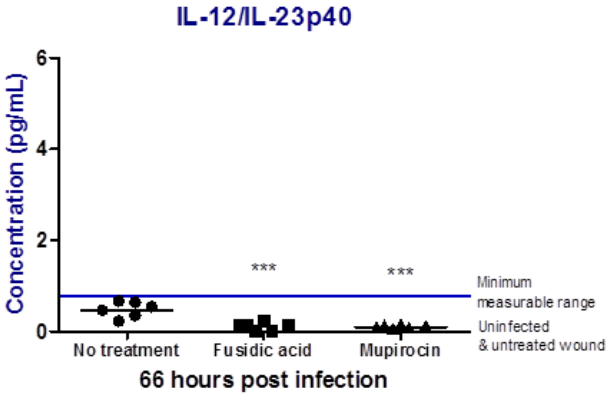

TNF-α
MIP-1β
TARC
IL-15
IL-7
IL-2
IL-4
Concentration (pg/mL)
100
10
1
0.1
ns
*
**
***
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

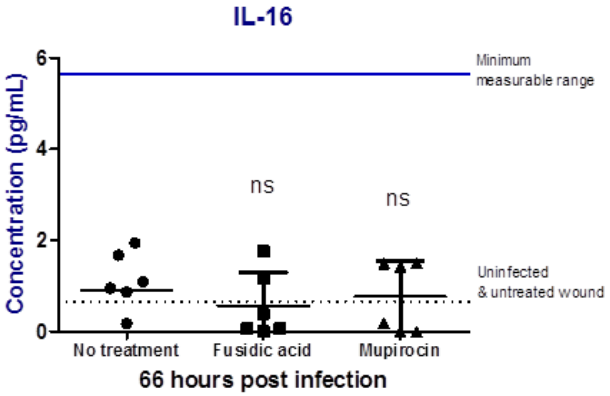

IL-8 (HA)
Concentration (pg/mL)
1000000
100000
10000
1000
100
ns
**
Maximum measurable range
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

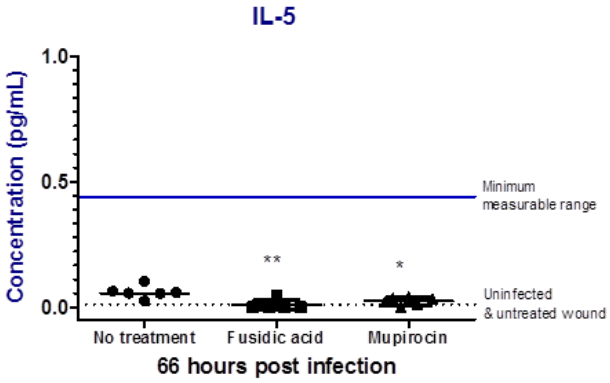

IL-8
Concentration (pg/mL)
1000000
100000
10000
1000
100
ns
ns
Uninfected & untreated wound
Maximum measurable range
No treatment
Fusidic acid
Mupirocin
66 hours post infection

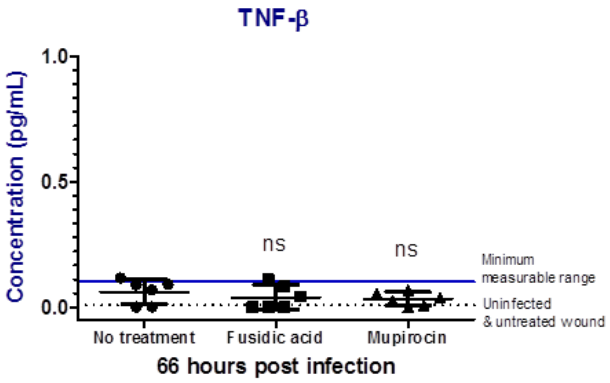

MCP-1
Concentration (pg/mL)
10000
1000
100
ns
ns
Uninfected & untreated wound
Maximum measurable range
No treatment
Fusidic acid
Mupirocin
66 hours post infection

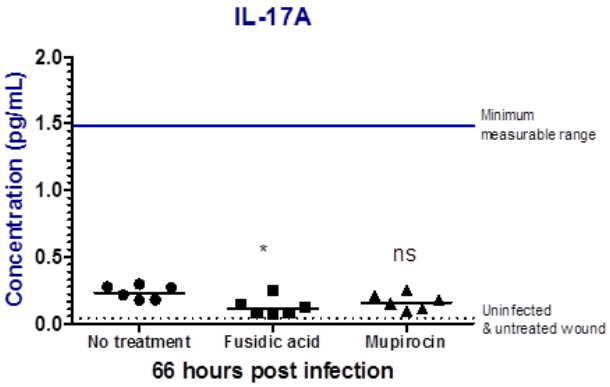

IL6
Concentration (pg/mL)
10000
1000
100
ns
ns
Uninfected & untreated wound
Maximum measurable range
No treatment
Fusidic acid
Mupirocin
66 hours post infection


IL-12/IL-23p40
Concentration (pg/mL)
6
4
2
0
***
***
Minimum measurable range
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

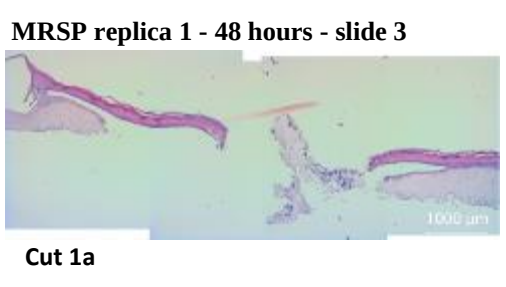

IL-16
Concentration (pg/mL)
6
4
2
0
ns
ns
Minimum measurable range
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

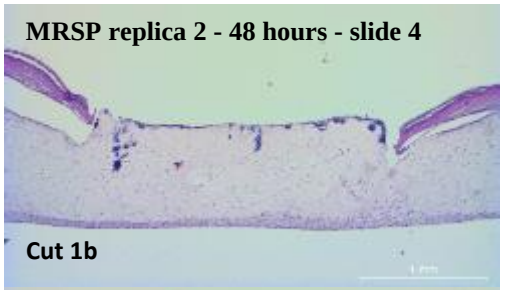

IL-5
Concentration (pg/mL)
1.0
0.5
0.0
**
*
Minimum measurable range
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

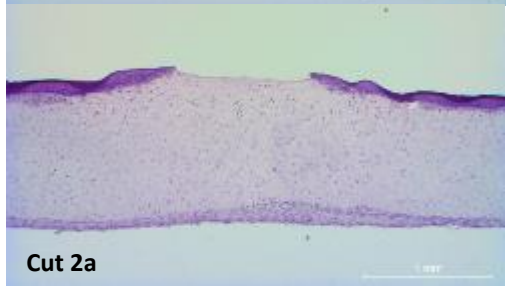

TNF-β
Concentration (pg/mL)
1.0
0.5
0.0
ns
ns
Minimum measurable range
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

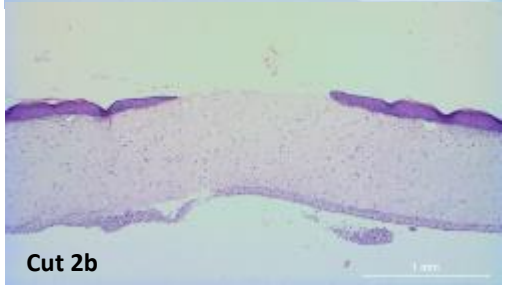

IL-17A
Concentration (pg/mL)
2.0
1.5
1.0
0.5
0.0
*
ns
Minimum measurable range
Uninfected & untreated wound
No treatment
Fusidic acid
Mupirocin
66 hours post infection

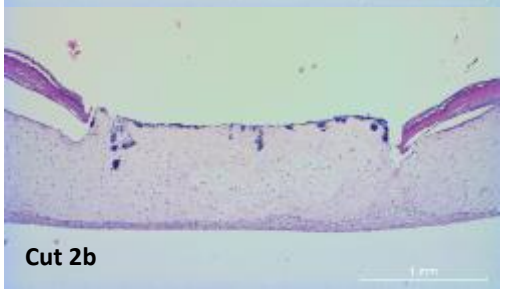
Appendix C – Bacterial load for different Staphylococcal strains in an *in vitro* skin model – Histology

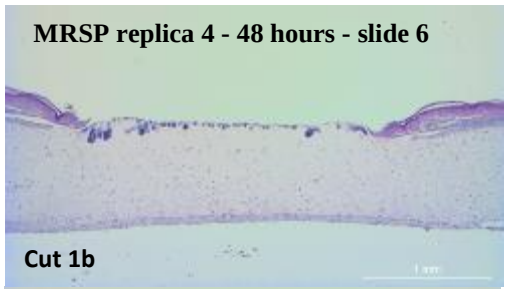


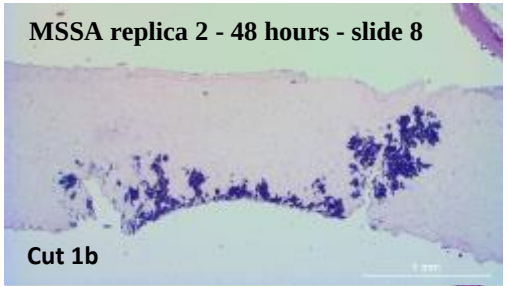


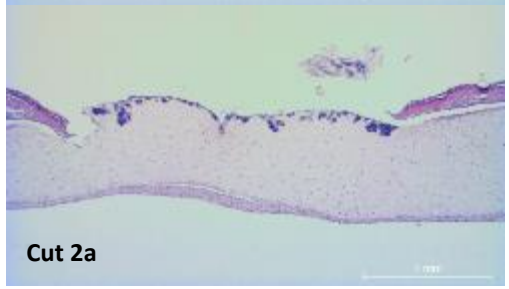


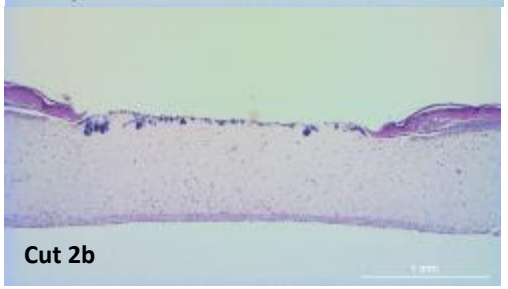


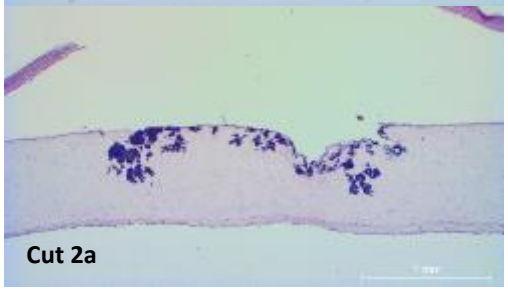


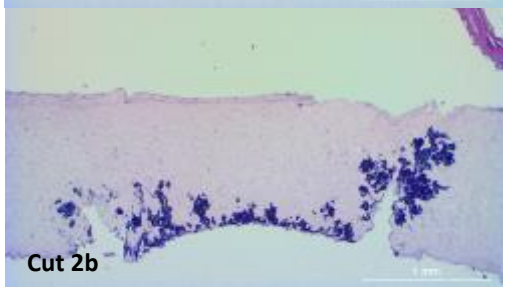


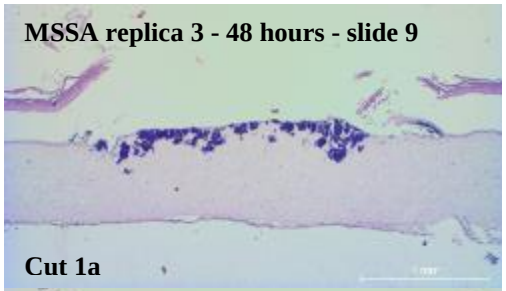


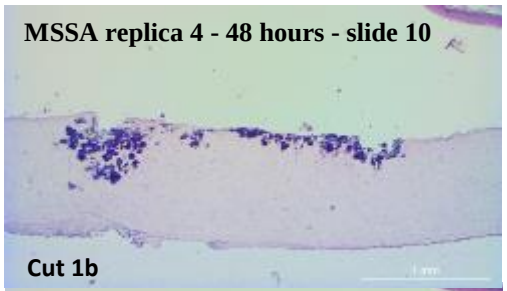


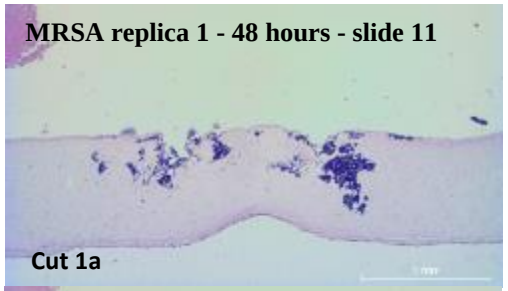


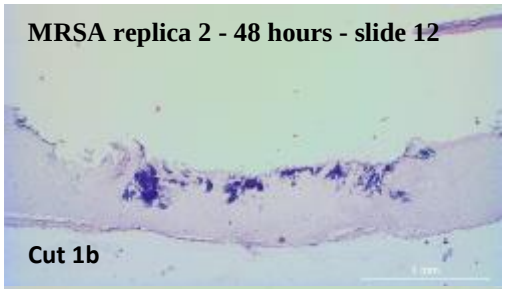


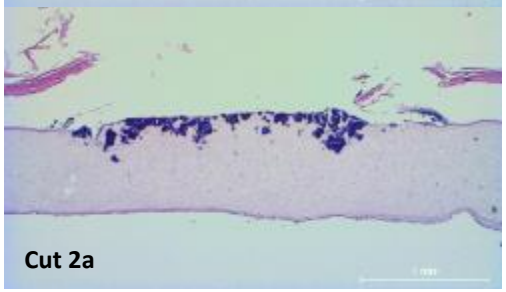


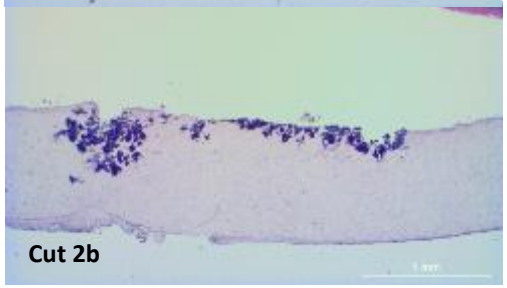

MSSA replica 3 - 48 hours - slide 9
Cut 1a

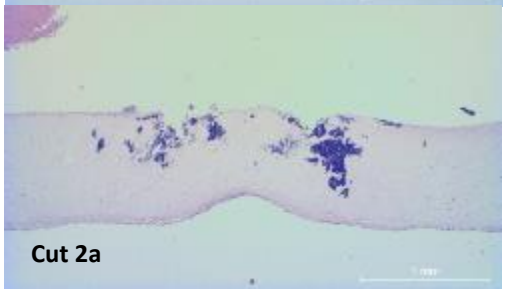

MSSA replica 4 - 48 hours - slide 10
Cut 1b

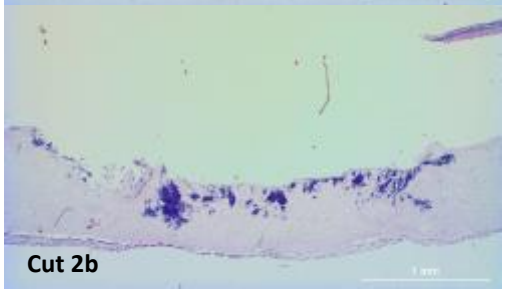

MRSA replica 1 - 48 hours - slide 11
Cut 1a

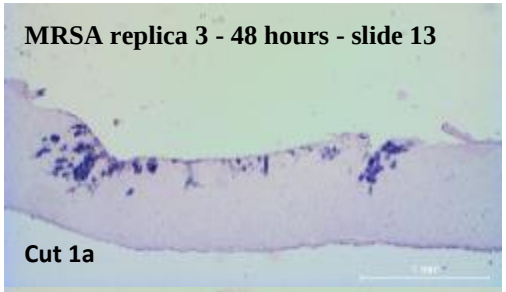

MRSA replica 2 - 48 hours - slide 12
Cut 1b

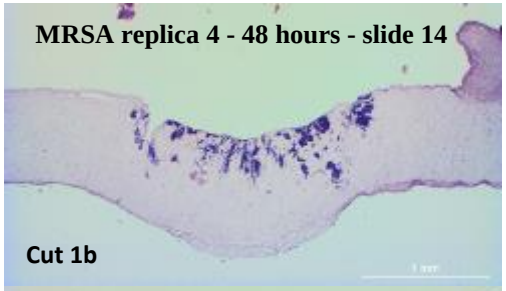

Cut 2a


Cut 2b

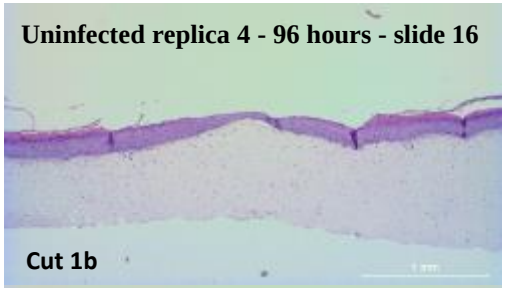

Cut 2a

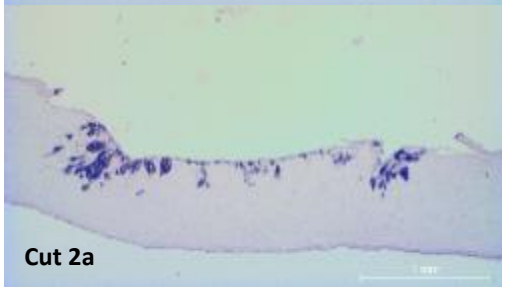

Cut 2b

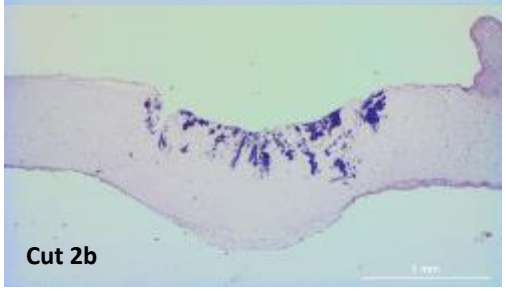

MRSA replica 3 - 48 hours - slide 13
Cut 1a

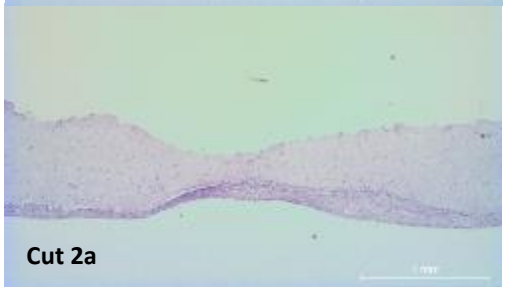

MRSA replica 4 - 48 hours - slide 14
Cut 1b

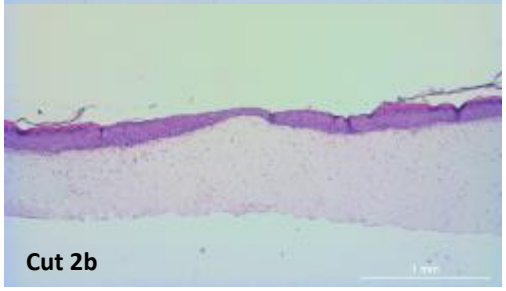

Uninfected replica 3 - 96 hours - slide 15
Cut 1a

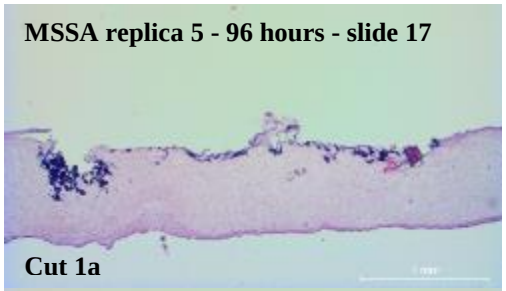

Uninfected replica 4 - 96 hours - slide 16
Cut 1b

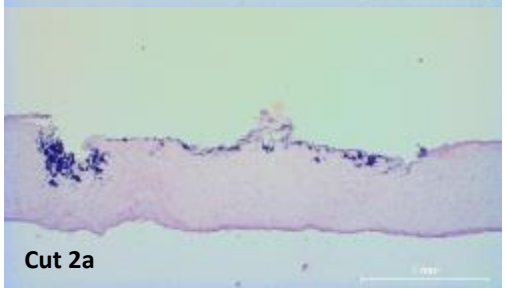

Cut 2a

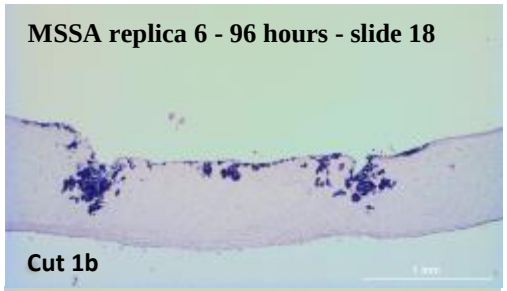

Cut 2b

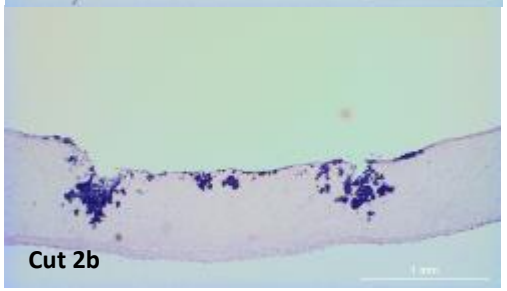

Cut 2a

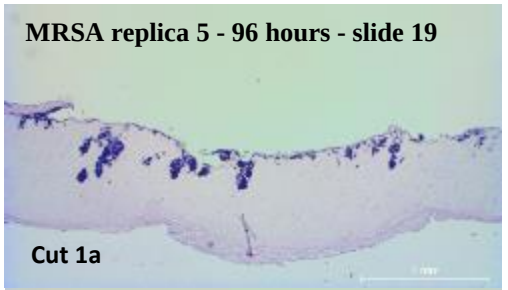

Cut 2b

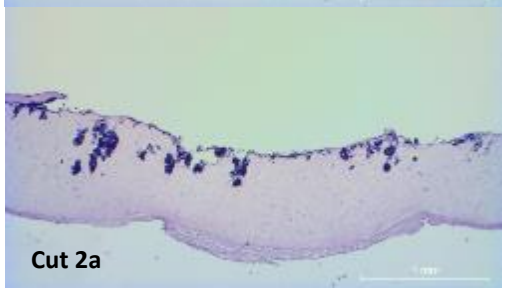

MSSA replica 5 - 96 hours - slide 17
Cut 1a

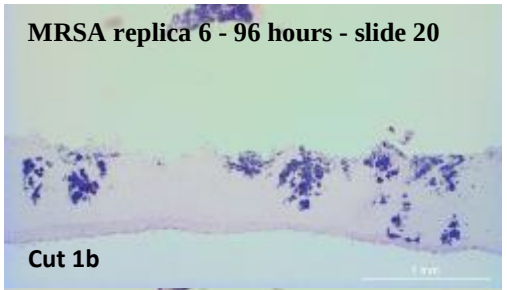

Cut 2a

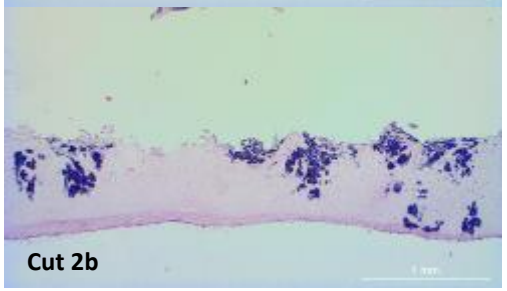

MSSA replica 6 - 96 hours - slide 18
Cut 1b


Cut 2b

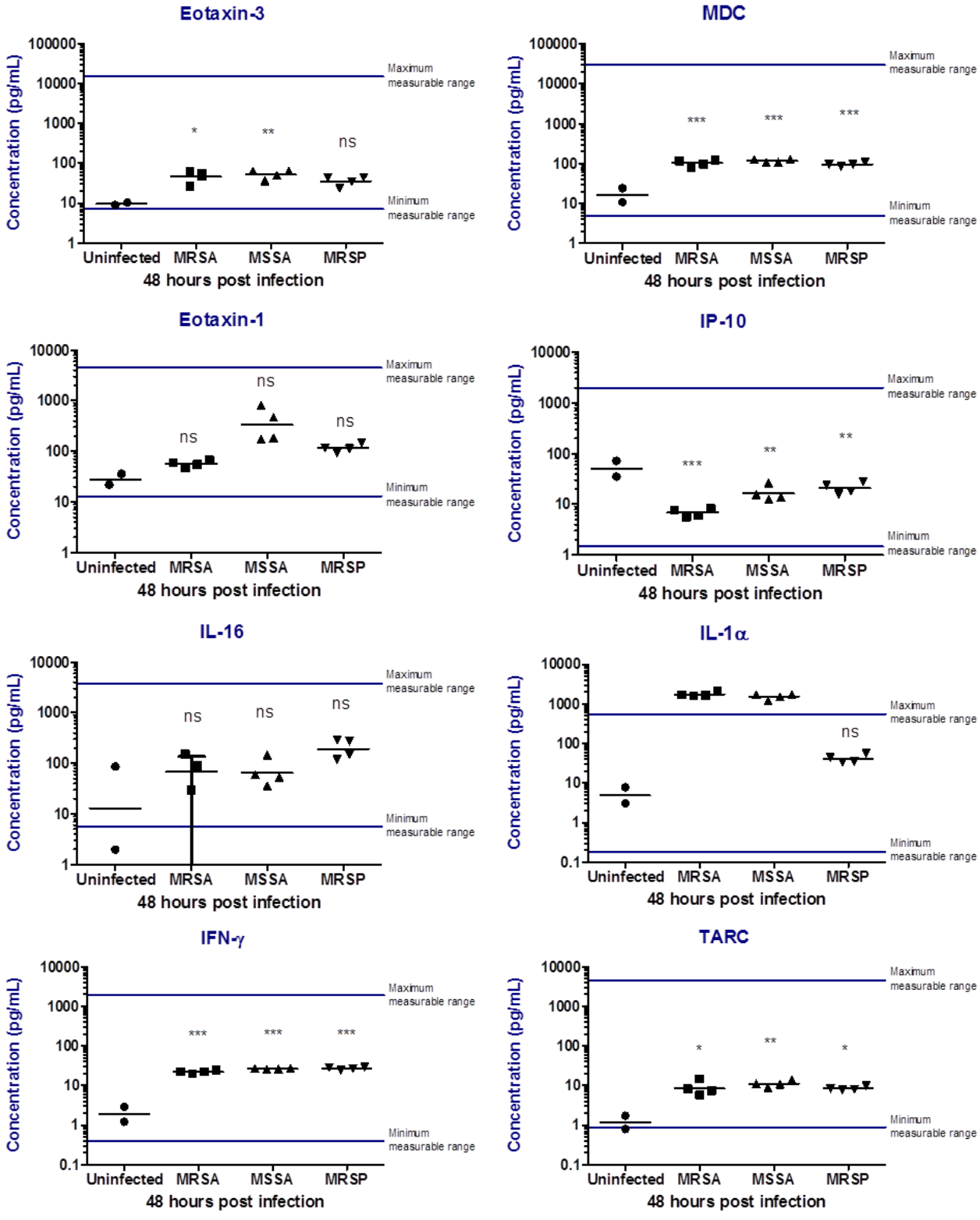

MRSA replica 5 - 96 hours - slide 19
Cut 1a

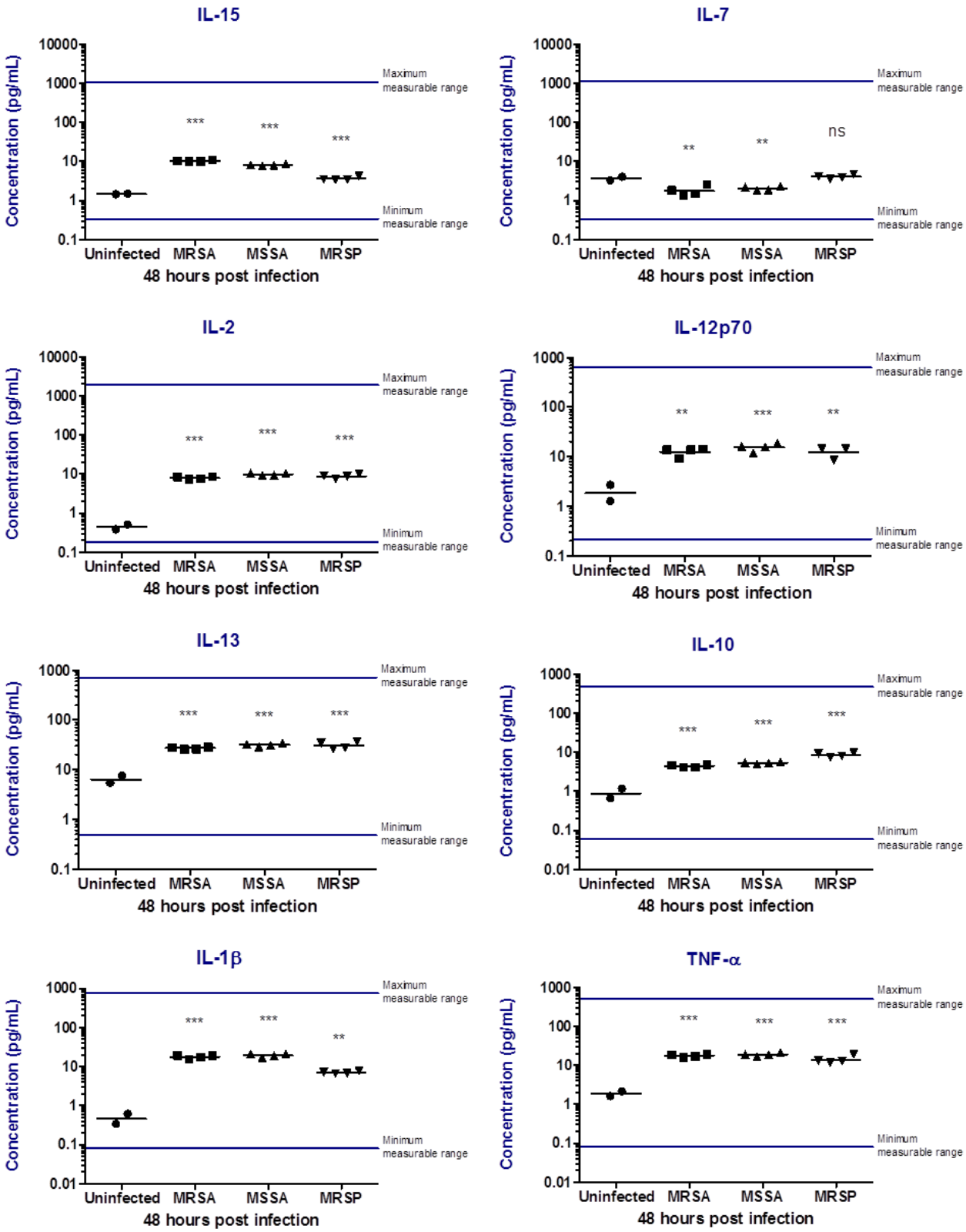

Cut 2a

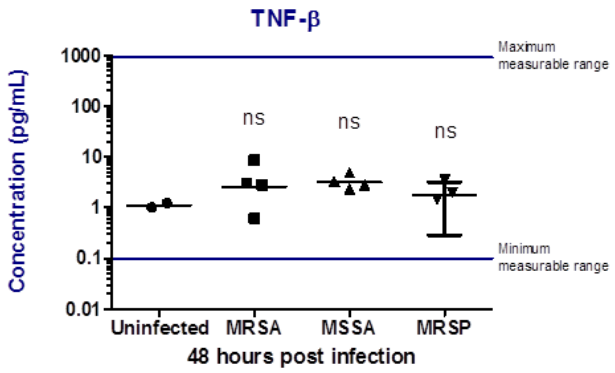

MRSA replica 6 - 96 hours - slide 20
Cut 1b

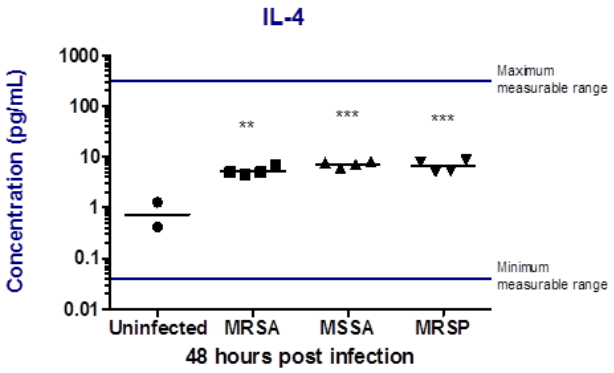

Cut 2b

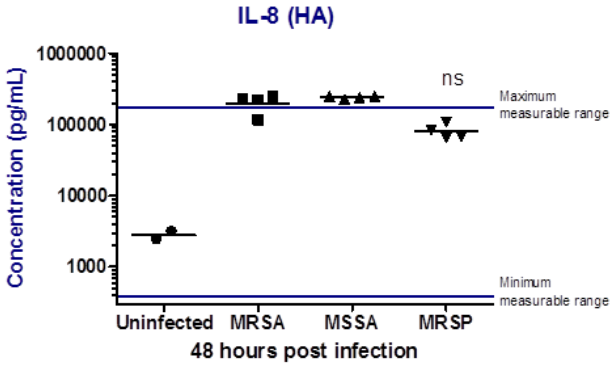
Appendix D – Bacterial load for different Staphylococcal strains in an *in vitro* skin model – Cytokines and chemokines

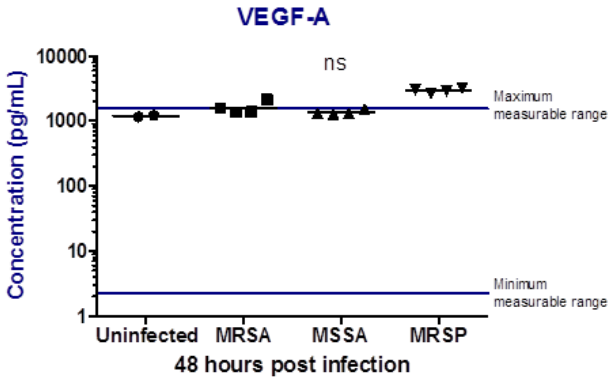


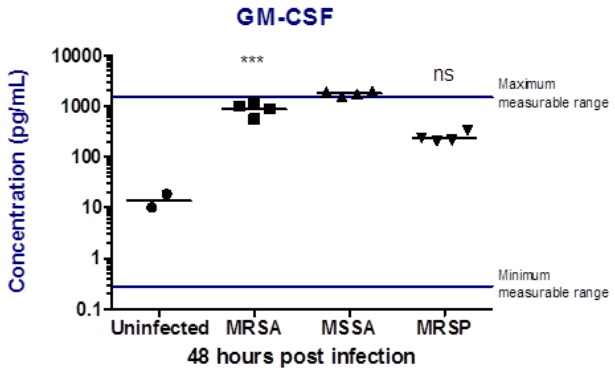

IL-15
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
***
***
***
Uninfected
MRSA
MSSA
MRSP
48 hours post infection
IL-7
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
**
**
ns
Uninfected
MRSA
MSSA
MRSP
48 hours post infection
IL-2
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
***
***
***
Uninfected
MRSA
MSSA
MRSP
48 hours post infection
IL-12p70
Concentration (pg/mL)
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
**
***
**
Uninfected
MRSA
MSSA
MRSP
48 hours post infection
IL-13
Concentration (pg/mL)
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
***
***
***
Uninfected
MRSA
MSSA
MRSP
48 hours post infection
IL-10
Concentration (pg/mL)
1000
100
10
1
0.1
0.01
Maximum measurable range
Minimum measurable range
***
***
***
Uninfected
MRSA
MSSA
MRSP
48 hours post infection
IL-1β
Concentration (pg/mL)
1000
100
10
1
0.1
0.01
Maximum measurable range
Minimum measurable range
***
***
**
Uninfected
MRSA
MSSA
MRSP
48 hours post infection
TNF-α
Concentration (pg/mL)
1000
100
10
1
0.1
0.01
Maximum measurable range
Minimum measurable range
***
***
***
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

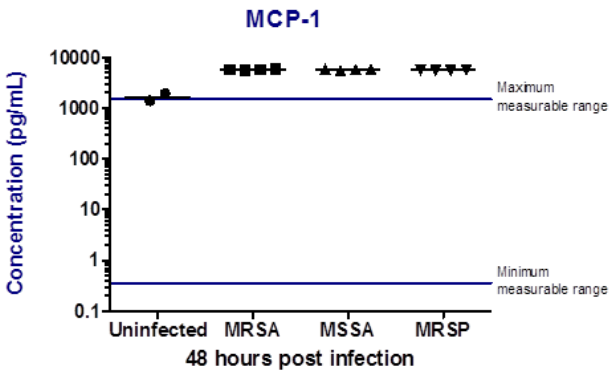

TNF-β
Concentration (pg/mL)
1000
100
10
1
0.1
0.01
ns
ns
ns
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

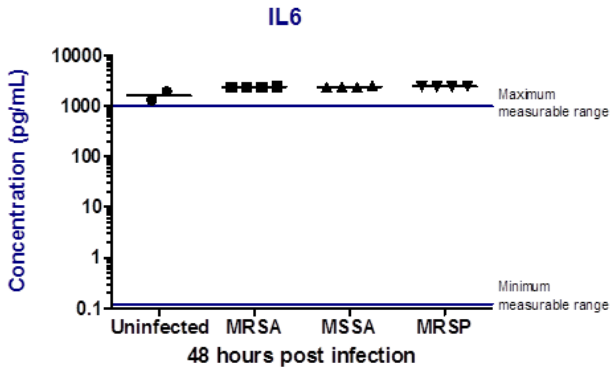

IL-4
Concentration (pg/mL)
1000
100
10
1
0.1
0.01
**
***
***
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

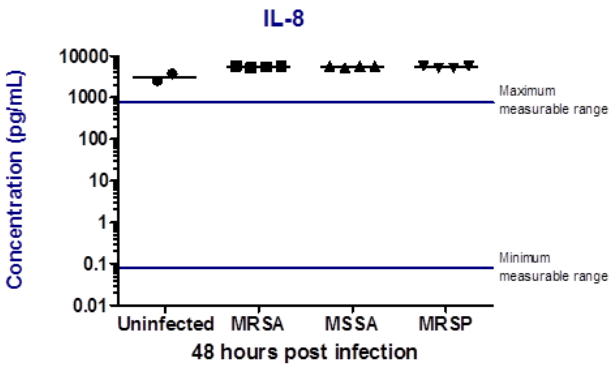

IL-8 (HA)
Concentration (pg/mL)
1000000
100000
10000
1000
ns
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

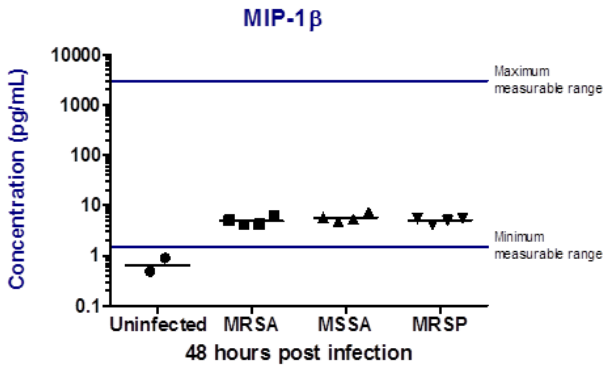

VEGF-A
Concentration (pg/mL)
10000
1000
100
10
1
ns
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

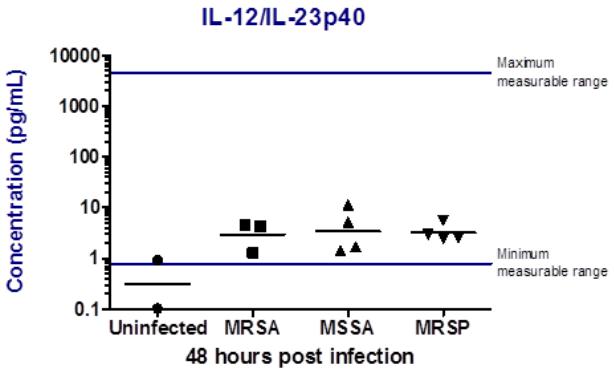

GM-CSF
Concentration (pg/mL)
10000
1000
100
10
1
0.1
***
ns
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

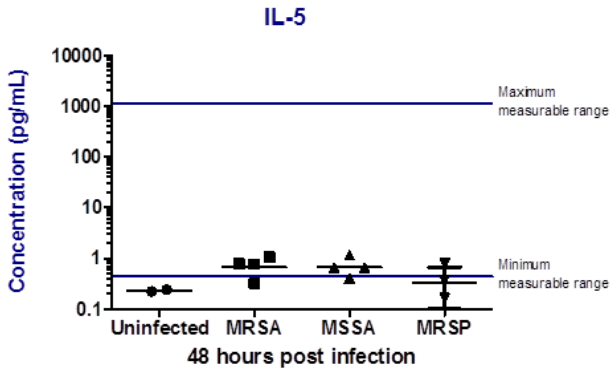

MCP-1
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

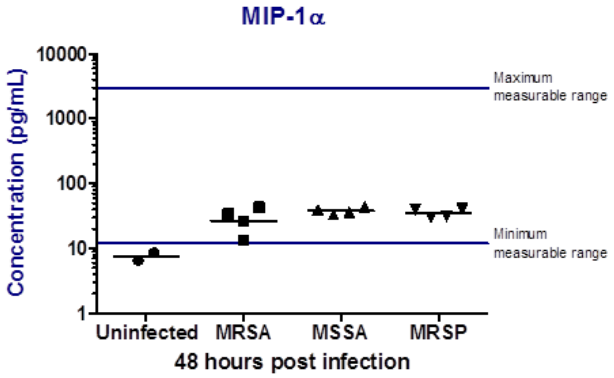

IL6
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

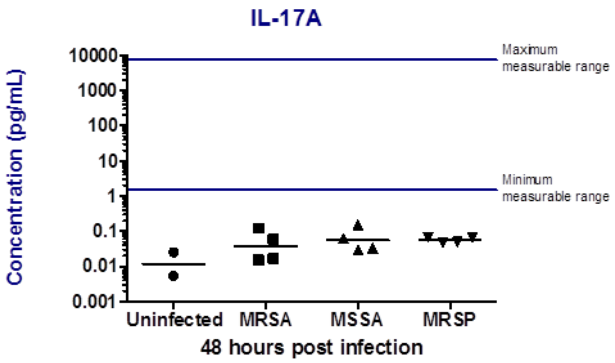

IL-8
Concentration (pg/mL)
10000
1000
100
10
1
0.1
0.01
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

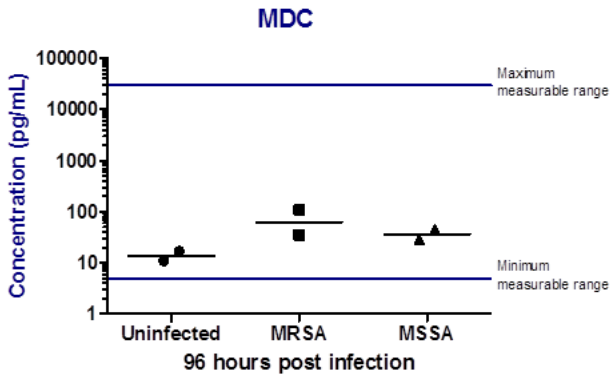

MIP-1β
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

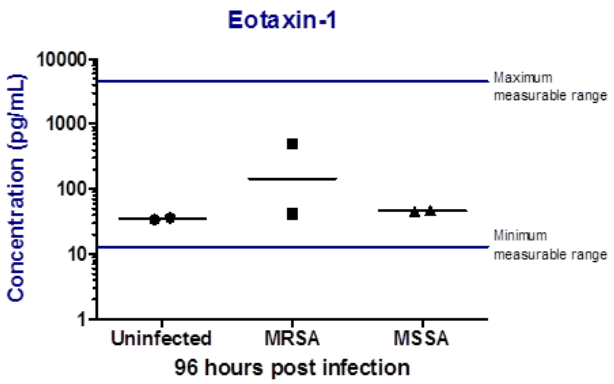

IL-12/IL-23p40
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

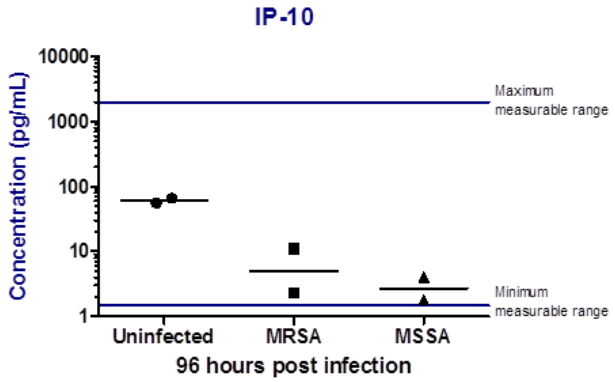

IL-5
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

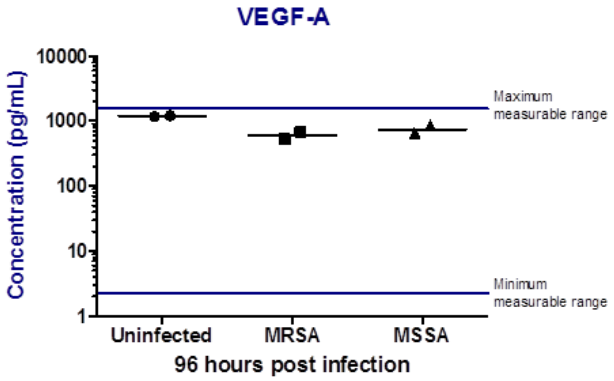

MIP-1α
Concentration (pg/mL)
10000
1000
100
10
1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

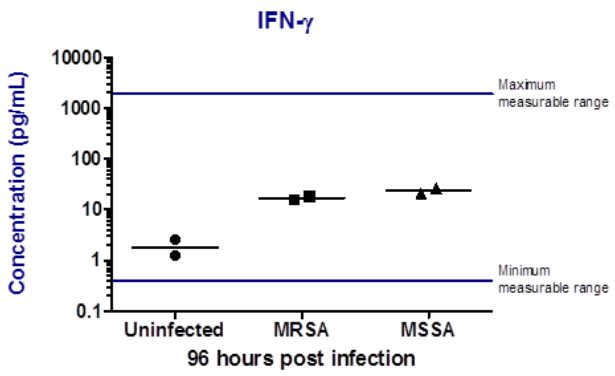

IL-17A
Concentration (pg/mL)
10000
1000
100
10
1
0.1
0.01
0.001
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
MRSP
48 hours post infection

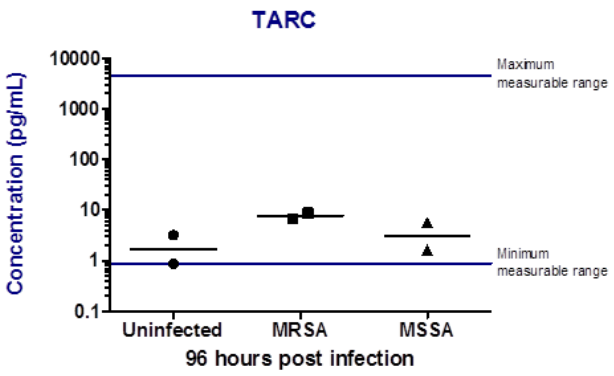

MDC
Concentration (pg/mL)
100000
10000
1000
100
10
1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

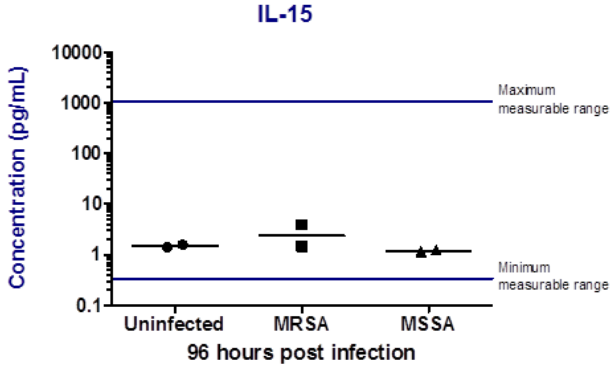

Eotaxin-1
Concentration (pg/mL)
10000
1000
100
10
1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

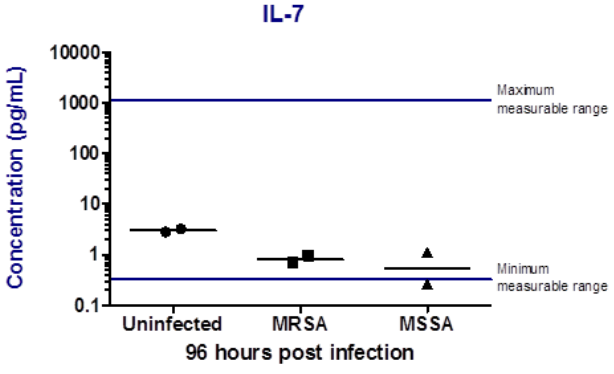

IP-10
Concentration (pg/mL)
10000
1000
100
10
1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

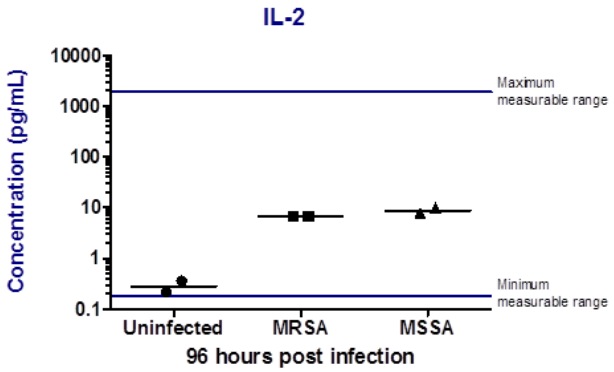

VEGF-A
Concentration (pg/mL)
10000
1000
100
10
1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

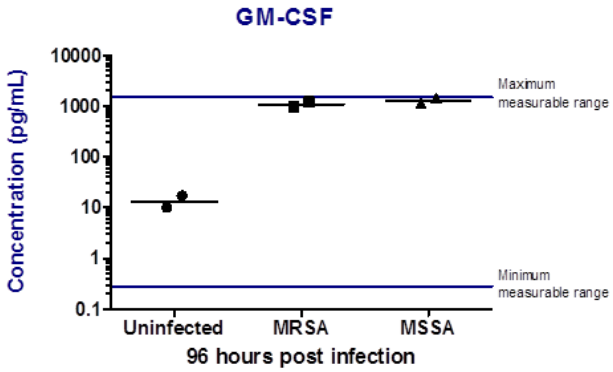

IFN-γ
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

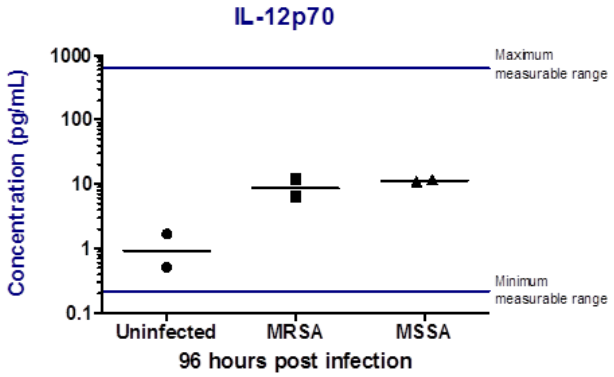

TARC
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

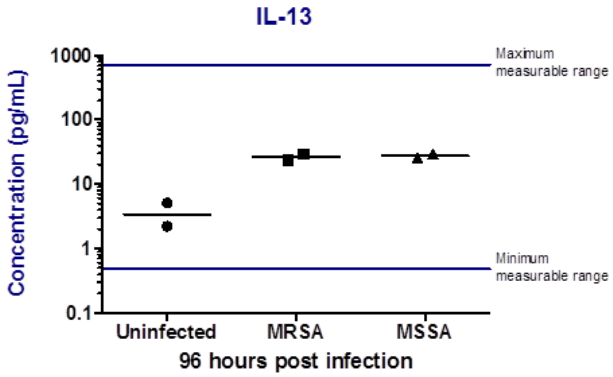

IL-15
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

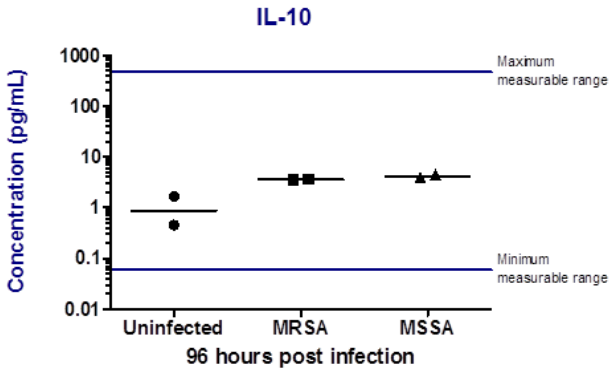

IL-7
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

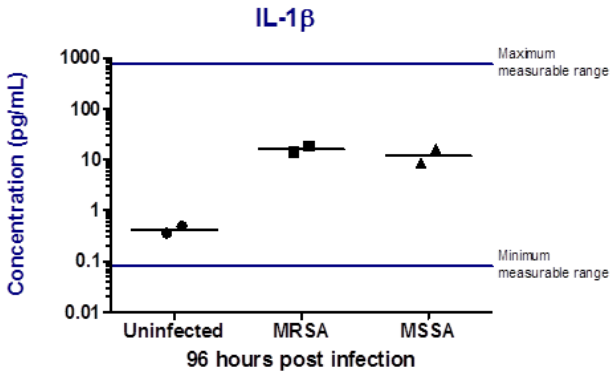

IL-2
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

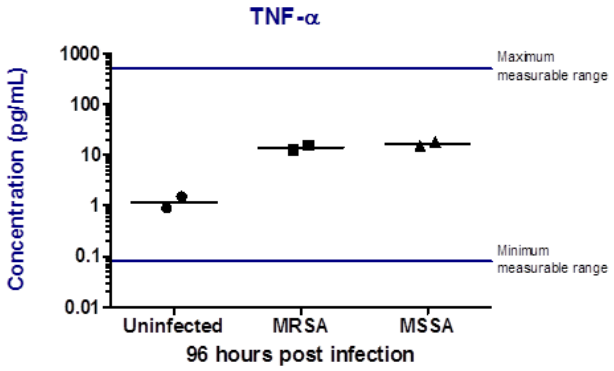

GM-CSF
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

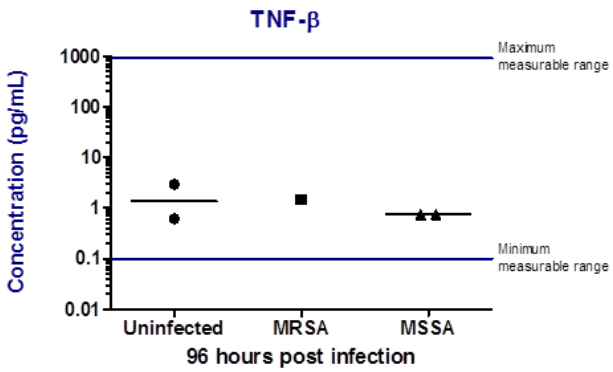

IL-12p70
Concentration (pg/mL)
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

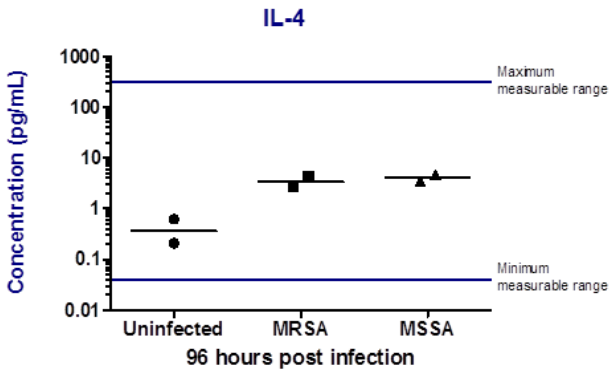

IL-13
Concentration (pg/mL)
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

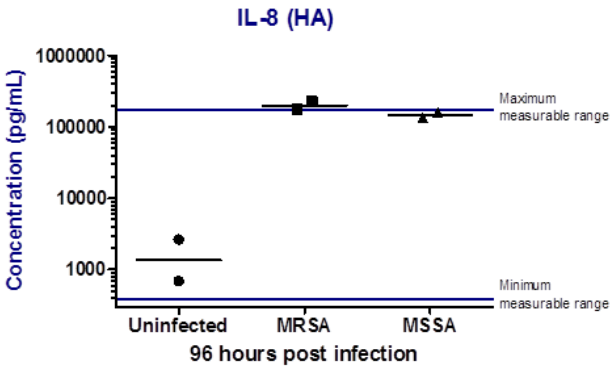

IL-10
Concentration (pg/mL)
1000
100
10
1
0.1
0.01
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

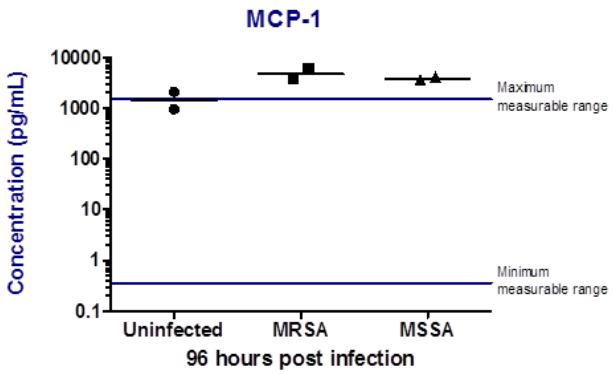

IL-1β
Concentration (pg/mL)
1000
100
10
1
0.1
0.01
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

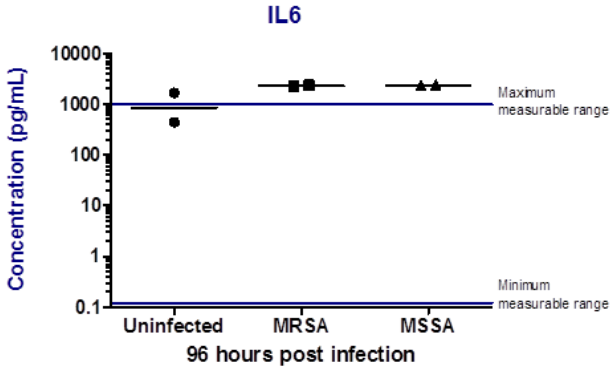

TNF-α
Concentration (pg/mL)
1000
100
10
1
0.1
0.01
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

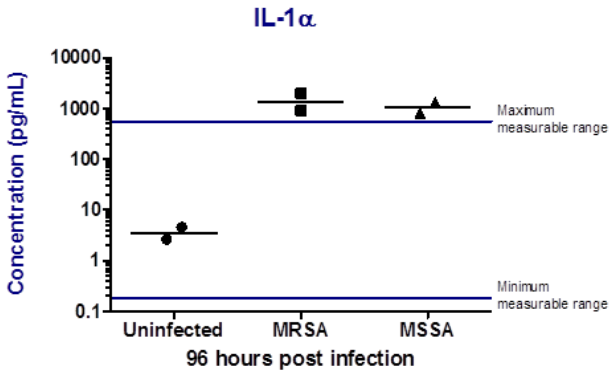

TNF-β
Concentration (pg/mL)
1000
100
10
1
0.1
0.01
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

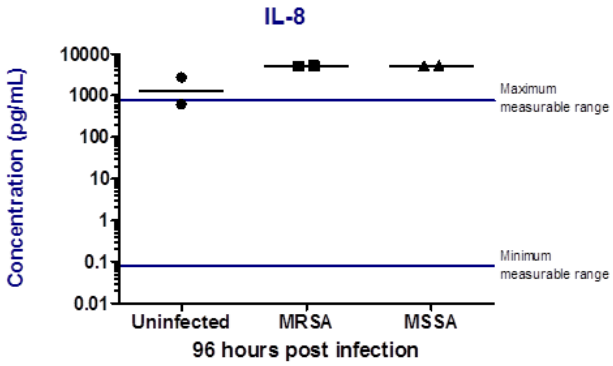

IL-4
Concentration (pg/mL)
1000
100
10
1
0.1
0.01
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

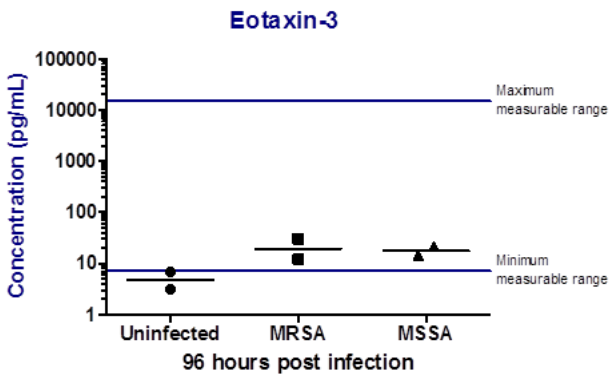

IL-8 (HA)
Concentration (pg/mL)
1000000
100000
10000
1000
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

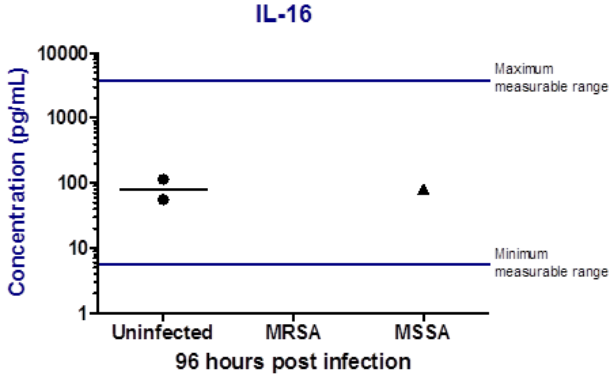

MCP-1
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

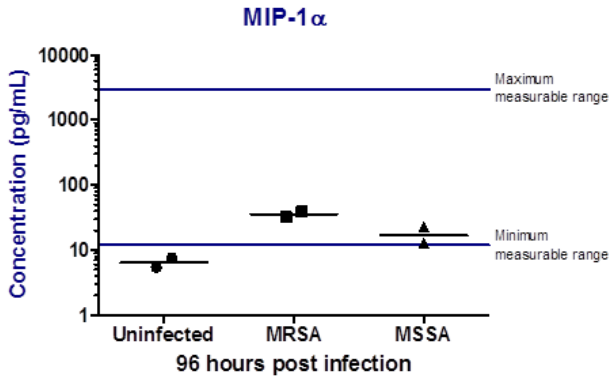

IL6
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

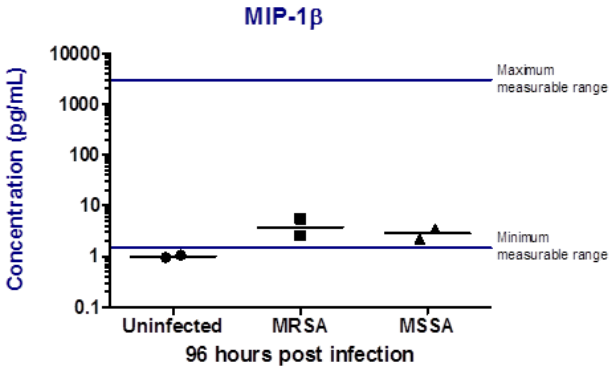

IL-1α
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

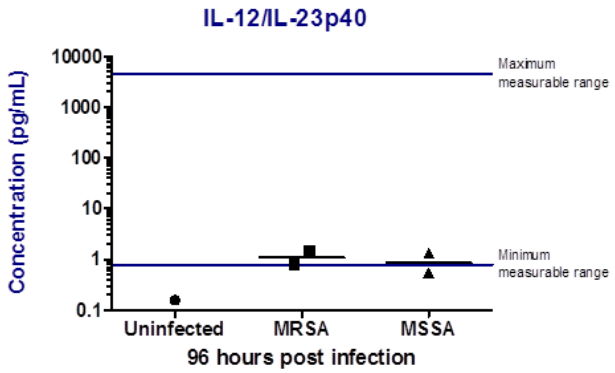

IL-8
Concentration (pg/mL)
10000
1000
100
10
1
0.1
0.01
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

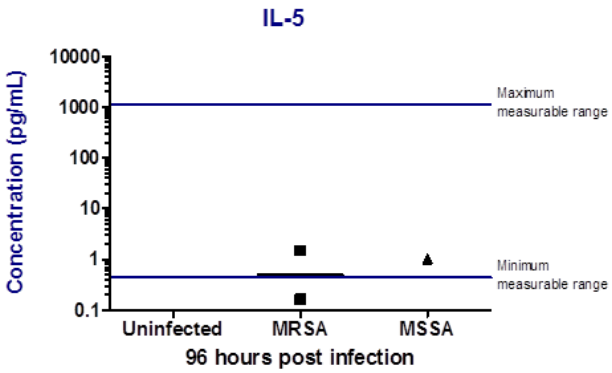

Eotaxin-3
Concentration (pg/mL)
100000
10000
1000
100
10
1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection

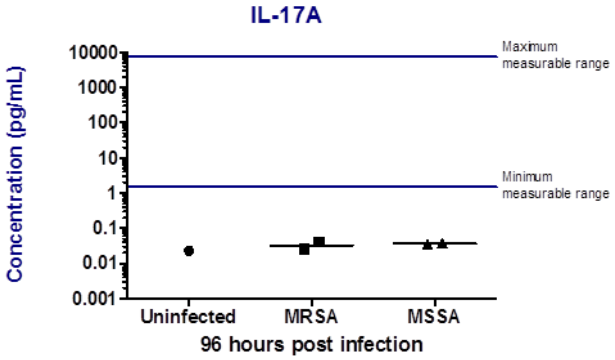

IL-16
Concentration (pg/mL)
10000
1000
100
10
1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection


MIP-1α
Concentration (pg/mL)
10000
1000
100
10
1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection


MIP-1β
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection


IL-12/IL-23p40
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection


IL-5
Concentration (pg/mL)
10000
1000
100
10
1
0.1
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection


IL-17A
Concentration (pg/mL)
10000
1000
100
10
1
0.1
0.01
0.001
Maximum measurable range
Minimum measurable range
Uninfected
MRSA
MSSA
96 hours post infection