FINAL REPORT

EUPR_010_101101 v1

Determination of the *in vitro* Efficacy of CHD-FA against Multi-Resistant Enterobacteriaceae and Mycobacteria

(Proposal: EUP_010_101101 V1.0)

FOR

Fulhold Limited

CONFIDENTIAL

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1.0 SUMMARY

Euprotec assessed the *in vitro* efficacy of CHD-FA for activity against multi-resistant Enterobacteriaceae and non-tuberculous *Mycobacterium*. CHD-FA was stored in the dark at room temperature following delivery.

2.0 METHODS

2.1 Strains

Susceptibility tests were performed on a range of multi-resistant Enterobacteriaceae and non-tuberculous *Mycobacterium*: Details of the strains used are outlined in table 1a and 1b.

2.2 Revival and Growth of the Strains

All strains were recovered from long-term storage at -80^{o}C by sub-culturing onto Cled agar plates, except for *Mycobacterium smegmatis* which was sub-cultured on to Middlebrook 7H10 agar. Plates were then incubated in air at 35-37^{o}C for 24 hours, except for *Mycobacterium smegmatis* which was incubated at 30^{o}C for 72 hours. Following visual checks to ensure purity and appropriate colony characteristics, isolates were deemed suitable for use.

Table 1a: NDM-1 and KPC containing Enterobacteriaceae strains (study 1)

SPECIES	Number	STRAIN	COMMENTS
Klebsiella pneumoniae	ATCC BAA 2146	New Delhi metallo-beta-lactamase (NDM-1) positive	bla_{KPC} negative by PCR bla_{NDM} positive by PCR
Klebsiella pneumoniae	NCTC1 3443	New Delhi metallo-beta-lactamase (NDM-1) positive	N/A
Klebsiella pneumoniae	ATCC BAA 1705	Modified Hodge Test (MHT) positive control designation	Strain produces *K. pneumoniae* carbapenemase (KPC) bla_{KPC} positive by PCR
Klebsiella pneumoniae	ATCC 700603	ESBL control strain	produces beta-lactamase SHV-18
Escherichia coli	Clinical 7	Clinical strain	ESBL Multi resistant strain
Escherichia coli	ATCC 25922	CLSI control strain	Susceptible isolate

Table 1b: Non-tuberculous Mycobacterium strains (study 2)

SPECIES	Number	COMMENTS
Mycobacterium smegmatis	ATCC 607	Validated in comparison assays with *M. tuberculosis*
Mycobacterium smegmatis	ATCC 19420	*Mycobacterium* Susceptibility control strain
Staphylococcus. aureus	ATCC 29213	CLSI Control strain for study

2.3 Preparation of the Inoculum

The inocula for each strain were prepared by picking 5-10 distinct colonies from the culture plates and suspending them in 3ml of sterile saline. The inoculum was resuspended by vigorous shaking on a vortex mixer for 15s. The turbidity was then adjusted to McFarland standard 0.5 (1-5 x 10^6 CFU/mL.). The inoculum was further diluted in Mueller Hinton broth for MIC tests to give a final inoculum in each well of 2-8 x 10^5 CFU/ml, except for *Mycobacterium smegmatis* which was further diluted in 7H9 broth with OADC Enrichment to give a final inoculum of 1.5 x 10^5 CFU/ml.

2.4 MIC Assay Conditions

MICs were tested in Mueller Hinton broth, except for *Mycobacterium smegmatis* MICs which was tested in 7H9 broth with OADC enrichment in accordance with the appropriate CLSI guidelines.

STEP 1: Addition of Test Article

a. The stock solution was provided by Fulhold as a 4% stock solution. This was further diluted in the appropriate media (Mueller Hinton broth or 7H9 broth with OADC enrichment) to give a top starting concentration of 2% in the assay. In addition, for each strain, a comparator control was included. The final concentration range for the comparator control (ciprofloxacin) was 0.03 -16 mg/L. 100µL of Mueller Hinton broth or 7H9 broth with OADC enrichment was dispensed into each well in columns 2-12. 200µL of the appropriate test compound solution (at 4%) was dispensed into each well in column 1.

b. 100µL aliquots were pipetted from column 1 wells and dispensed into column 2 with a multichannel pipette (± 2% coefficient of variation) thus diluting two-fold. 100 µL samples were then pipetted from column 2 wells and dispensed into column 3. The process was repeated through to column 10. The final 100 µL of diluted drug from column 10 was then discarded. Row 11 acted as a positive control (no drug or test article, organisms added), Row 12 acted as a negative control (no drug or test article, and no organisms added).

STEP 2: Addition of Bacterial Strains

100µL of the appropriate inoculum suspension in Mueller Hinton broth or 7H9 broth with OADC enrichment was added to the appropriate wells. This resulted in a well containing 200µL final volume (made up of 100µL diluted compound or diluents and 100µL of inoculum or broth alone).

STEP 3: Incubation of Assay Plates

All plates were incubated in the dark at 35-37^{o}C in air for 18-24 hours, except for the *Staphylococcus aureus* and *Mycobacterium smegmatis* combined plate which were incubated at 30^{o}C in air for 16-20 hours and 72 hours respectively.

STEP 4: Reading of Plates

Plates were read visually and spectrophotometrically (450nm) where possible, 20 or 72 hours post inoculation. Endpoints of 50%, 80% and 100% inhibition were determined (or CLSI interpretation endpoints following visual examination).

3.0 RESULTS

3.1 Visual MIC values (50%, 80% and 100% inhibition).

Table 2: Colour coded MIC efficacy data of CHD-FA against various Enterobacteriaceae and Mycobacteria strains

	Klebsiella pneumoniae ATCC BAA 2146			*Klebsiella pneumoniae* NCTC 13443			*Klebsiella pneumoniae* ATCC BAA 1705			*Klebsiella pneumoniae* ATCC700603			*Escherichia coli* Clinical strain 7			*Escherichia coli* ATCC 25922		
Compound	**100%**	**80%**	**50%**	**100%**	**80%**	**50%**	**100%**	**80%**	**50%**	**100%**	**80%**	**50%**	**100%**	**80%**	**50%**	**100%**	**80%**	**50%**
CHD-FA (%)	0.12	0.12	0.12	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Ciprofloxacin (μg/ml)	>16	>16	>16	>16	>16	>16	>16	>16	>16	0.25	0.25	0.25	0.06	0.06	0.06	≤0.03	≤0.03	≤0.03

	Mycobacterium smegmatis ATCC 607			*Mycobacterium smegmatis* ATCC 19420			*Staphylococcus aureus* ATCC 29213		
Compound	**100%**	**80%**	**50%**	**100%**	**80%**	**50%**	**100%**	**80%**	**50%**
CHD-FA (%)	0.06	0.06	0.06	0.06	0.06	0.06	0.12	0.06	0.03
Ciprofloxacin (μg/ml)	0.25	0.25	0.25	0.25	0.12	0.12	0.5	0.25	0.25

Colour Code: CHD_FA

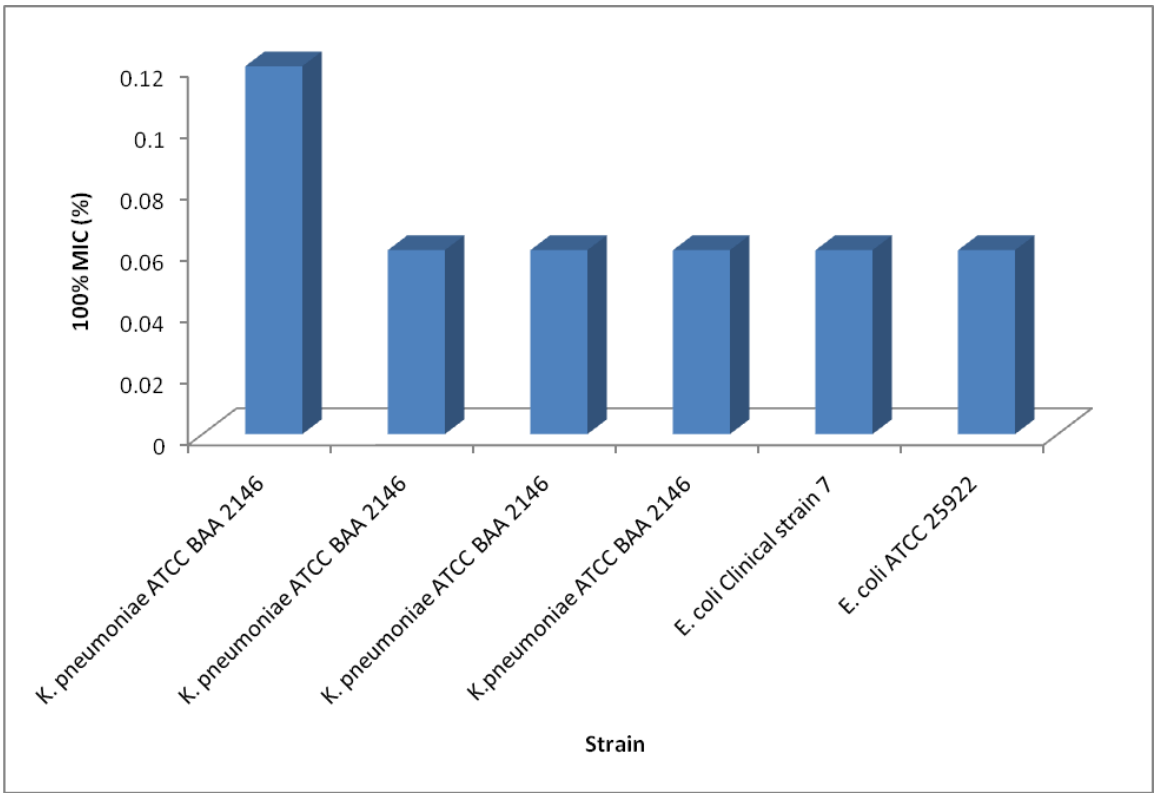
>2-2 %
1 %
0.5%
0.25-0.06 %
≤0.03%

Colour Code: Ciprofloxacin

>16-16 mg/L
8 mg/L
4 mg/L
2-0.5 mg/L
≤0.25mg/L

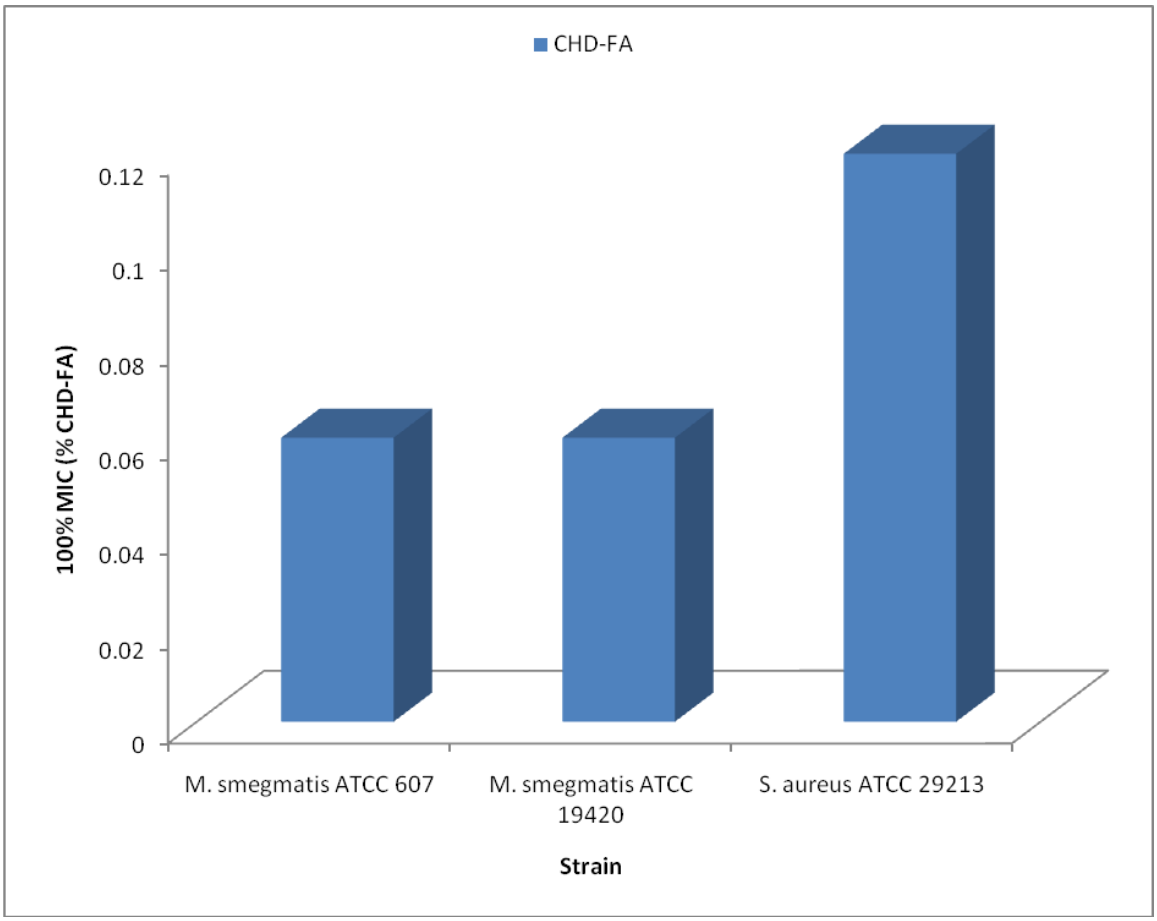
3.2 MIC efficacy of CHD-FA against NDM-1 and KPC containing Enterobacteriaceae strains

Figure 1: Effect of CHD-FA against NDM-1 and KPC containing Enterobacteriaceae strains is shown in figure 1.



3.3 MIC efficacy of CHD-FA against non-tuberculous Mycobacterium strains

Figure 2: Effect of CHD-FA against non-tuberculous *Mycobacterium* strains is shown in figure 2.



4.0. SUMMARY

- CHD-FA was effective against NDM-1, KPC and ESBL positive *Klebsiella pneumonia* and multi-resistant *E. coli* strains with a 100% MIC value of 0.06-0.12%.

- The ciprofloxacin control was not effective against both NDM-1 and KPC positive *Klebsiella pneumonia* strains with a MIC of >16 μg/ml. However, ciprofloxacin was effective against the *Klebsiella pneumonia* and *E. coli* ESBL positive strains.

- CHD-FA and ciprofloxacin were each effective against both strains of *Mycobacterium smegmatis* with a 100% MIC value of 0.06% and 0.25μg/ml respectively.

- The ciprofloxacin MICs against *Escherichia coli* and *Staphylococcus aureus* control strains were within CLSI guidelines.

- CHD-FA was highly effective against multi-resistant Gram negative bacilli including NDM-1 positive strains. CHD-FA was also highly effective against *Mycobacterium smegmatis*. All organisms were inhibited using ≤0.12% of CHD-FA

5.0. AUTHENTICATION STATEMENT

I, the undersigned, hereby declare that the findings provide a true and accurate record of the results obtained.

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Dr Peter Warn Date

Director

Euprotec Limited

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Dr Sandra Howsley Date

Projects Manager

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