# Final Report

**CONFIDENTIAL**

## A study to determine the virucidal efficacy of a test article against NIBRG-14 [H5N1] Influenza virus


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## 3 SUMMARY

The test article reduced the titre of avian Influenza NIBRG-14 (H5N1) virus by at least 1.25 $-\log_{10}$ $TCID_{50}$/ml (94.38%) at a test concentration of 90% (v/v) for the 30 second, 60 second, 5 minute and 10 minute time points.

The test article was observed to be toxic to the MDCK cell line at the test concentration for the $10^{-1}$ and $10^{-2}$ dilutions.

# 4 STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE REGULATIONS

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data to be valid.

- The United Kingdom Good Laboratory Practice Regulations 1999 Statutory Instrument No. 3106.
- The United Kingdom Good Laboratory Practice (Codification Amendments etc.) Regulations 2004 Statutory Instrument No. 994.
- OECD Principles of Good Laboratory Practice, (Revised 1997).

**Miss Elizabeth Moane**

Study Director

Retroscreen Virology Ltd.

# 5 QUALITY ASSURANCE STATEMENT

Quality Assurance has audited this report. The methods, practices and procedures reported herein are an accurate description of those employed at Retroscreen Virology Ltd during the course of the study. Observations and results presented in this final report form a true and accurate representation of the raw data generated during the conduct of the study at Retroscreen Virology Ltd.

**Mr. Jonathan Riley**

Quality Assurance

Retroscreen Virology Ltd.

# 6 INTRODUCTION

In the past century we have witnessed three pandemics of Influenza, of which the "Spanish flu" of 1918 was the largest pandemic of any infectious disease known to medical science (Oxford, J. S., 2000). The three strains which caused these pandemics belong to group A of the influenza viruses and, unlike the other two groups (B and C), this group infects a vast variety of animals (poultry, swine, horses, humans and other mammals).

Influenza A viruses continue to cause global problems, both economically and medically (Hayden, F. G. & Palese, P., 2000). Although children and younger adults experience the most cases of Influenza infection, severe illness is more common in the elderly or immuno-compromised individuals with chronic illnesses such as asthma, diabetes, kidney failure and heart disease. The annual epidemics of Influenza run from November to March in the Northern Hemisphere and from April to September in the Southern Hemisphere

The current global concern is the avian Influenza A H5N1 virus, which first demonstrated its ability to infect birds in China in 1997 and has since spread to other countries in South East Asia (Guan, Y. *et al.*, 2004; Peiris, J. S. *et al*., 2004), Europe and Africa (Enserink, M, 2006). Its ability to cause severe disease in birds has been documented by the World Health Organisation during a mild outbreak in South East Asian birds during 2003-2004. H5N1 mutates rapidly and is highly pathogenic. Its co-existence with other avian influenza viruses increases the likelihood of concurrent infections in birds. Such events would provide the 'mixing vessel' for the emergence of a novel subtype with sufficient avian genes to be easily transmitted between avian species, which would mark the start of an influenza epidemic (WHO Fact sheet).

With the recent news of a probable H5N1 pandemic the need to prevent any opportunities of transmission of the virus between avian species has risen. Disinfectants would be ideal for use in poultry farms or quarantine units to prevent species-to-species transmission of the virus. In addition, healthcare workers, research scientists and those handling infected animals would benefit from a disinfectant against the H5N1 virus.

# 7 AIMS OF THE STUDY

The primary objective was to determine the virucidal activity of the test article against Avian Influenza A NIBRG-14 [H5N1] virus at one concentration for four incubation time points.

The secondary objective was to assess any cytotoxic potential of the test article on the MDCK cell line.

# 8 MATERIALS AND METHODS

## 8.1 Test articles and control reference articles

### 8.1.1 Test articles

The test article was GAMA Healthcare Clinell disinfectant formula. The test article was assessed undiluted. In the virucidal assay, 9 volumes of test article were added to one volume of virus, yielding the final test concentration of 90% v/v.

The test article was supplied by the Sponsor as an undiluted preparation.

The characteristics of the test article were the responsibility of the Sponsor.

### 8.1.2 Control reference articles

The control(s) utilised in the toxicity assay are:

- Cell only control: untreated cell. This was a negative control for tCPE (toxic cytopathic effect) and was also an indicator of cell quality. As standard infection media was used as a diluent, this was also the diluent control for the assay.

The controls utilised in the virucidal assay are:

- Cell only control: cells not infected with virus. This is a negative control for vCPE (viral cytopathic effect) and was also an indicator of cell quality.
- Diluent control: cells infected with virus that was pre-treated with standard infection media for the specified time. This was a negative control for the test articles and assessed any antiviral effects of the diluent.
- Antiviral control: cells infected with virus that was pre-treated with citrate buffer at pH3.5. This was a positive control for comparison with the test article.

The cells of the controls used in each assay were incubated with standard cell infection media.

## 8.2 Further information

### 8.2.1 Cells and viruses

The cells used in this study were MDCK cells and were supplied from the Retroscreen Virology Ltd. cell bank.

The virus used in this study was Avian Influenza A NIBRG-14 [H5N1] virus. The NIBRG-14 virus is a re-assortant virus between the A-PR8 and Influenza A/Vietnam viruses, created by NIBSC, UK.

The stock titre of avian Influenza NIBRG-14 virus (AL:870) was approximately 7.25 $\log_{10}$ $TCID_{50}$/ml. Before use in the virucidal assay, the stock virus was diluted 1/10 (v/v) in MDCK infection media to obtain a titre of approximately 6.25 $\log_{10}$ $TCID_{50}$/ml.

This virus was diluted a further 10-fold when it was added to the test article (section 8.5) to form the reaction mixture. Thus, the expected TCID of the reaction mixture is 5.25 $\log_{10}$ $TCID_{50}$/ml +/- 1.00 $\log_{10}$ $TCID_{50}$/ml.

The total dilution of the virus, after termination, on addition to the plates was 1/1000 (v/v). To ensure consistency, the control virus was also diluted 1/1000 (v/v) before addition to the plate.

#### 8.2.2 Test Variables

The test articles were tested for virucidal activity by incubation with the virus for the following time point(s):

- 30 seconds
- 1 minute
- 5 minutes
- 10 minutes

#### 8.2.3 Study time lines

The study protocol was signed off on 20 Feb 2006

The study was initiated on 23 Feb 2006 and completed on 10 Mar 2006.

## 8.3 Preparation of MDCK cells

MDCK cells (100µl/well) were seeded onto 96-well plates at a density of ~$5x10^4$cells/ml. The cells were incubated at 37°C and 5% $CO_2$ for ~24 hours.

The plates were washed twice with PBS (100µl/well) before use in either the cytotoxicity or virucidal assays.

## 8.4 Cytotoxicity assay

The test articles were diluted in the same way as in the virucidal assay (i.e. 40µl of MDCK infection media was added to 360µl of the undiluted test article and then the total volume of 400µl was added to 3.6mls of MDCK infection media).

Each test article dilution was titrated, in triplicate following a 10-fold dilution series from neat (111µl in the first well) across a 96-well plate of MDCK cells seeded at ~$5x10^4$ cells/ml. The plate was incubated at 37°C and 5% $CO_2$ for ~1 hour.

After incubation, the cell monolayer was washed twice with PBS (100µl/well) and fresh standard infection media (100µl/well) was added. The plate was incubated at 37°C and 5% $CO_2$ for ~4 hours.

After incubation, the plates were observed for tCPE (toxic cytopathic effect).

## 8.5 Virucidal assay

Virus (40µl) was added to the test article (360µl) and incubated at room temperature for the specified time points (section 8.2.2).

After incubation, the reaction was terminated by the addition of standard infection media (3.6ml), which diluted the reaction 10-fold.

The terminated mixture was titrated, in quadruplicate, across a 96-well plate of MDCK cells following a 10-fold dilution series.

The cells were incubated for ~1 hour at 37°C, 5% $CO_2$. After incubation, the supernatant was discarded from the plates and the cell monolayer washed twice with PBS (100µl) and fresh standard infection media (100µl) added.

The cells were incubated for 3 days at 37°C, 5% $CO_2$. After incubation the Haemagglutination assay (HA) was performed on the supernatant to determine the endpoint of the titrations.

The titrations were carried out in accordance to the Retroscreen Virology Ltd. SOP VA016-04 (Titration of Samples Containing Influenza Virus on MDCK Cells).

The HA assay was carried out in accordance to the Retroscreen Virology Ltd. SOP VA018-02 (The Haemagglutination Assay).

# 9 RESULTS

## 9.1 Cytotoxicity Assay

The cytotoxicity assay (detailed in section 8.4) was used to determine if the test articles had any toxic effects on the MDCK cell line. The cells were observed for tCPE to determine any cytopathic effects caused by the test article. The tCPE observations are detailed in Table 1.

***Table 1: tCPE observations of the MDCK cell monolayer after treatment with the test articles and diluent contol (MDCK Infection Media)***

| Dilution ($10^x$) | MDCK Infection Media | | | GAMA Healthcare Clinell Disinfectant | | |
|---|---|---|---|---|---|---|
| **-1** | - | - | - | T | T | T |
| **-2** | - | - | - | T | T | T |
| **-3** | - | - | - | - | - | - |
| **-4** | - | - | - | - | - | - |
| **-5** | - | - | - | - | - | - |
| **-6** | - | - | - | - | - | - |
| **-7** | - | - | - | - | - | - |
| **Cell only** | - | - | - | - | - | - |

Key:

T = positive for tCPE
- = negative for tCPE

## 9.2 Virucidal Assay

The virucidal activity of GAMA Healthcare Clinell disinfectant against avian Influenza NIBRG-14 [H5N1] was assessed for four different time points. The results of this, determined from the Haemaglutination assay are detailed in Table 2. The virus control was observed to be within the expected $\log_{10}$ $TCID_{50}$/ml.

***Table 2: Reduction in virus titre of avian Influenza NIBRG-14 [H5N1] virus after treatment with the test article and positive control article (citrate buffer at pH3.5)***

| **Test reaction** | **Virus titre recovered** *(log10 TCID50/ml)* | | **Reduction in virus titre** | |
|---|---|---|---|---|
| | *Virus control* | *Test article* | *(-log10 TCID50/ml)* | *(%)* |
| Test article for 30 seconds | 4.75 | ≥2.0[A] | ≤2.75[A] | ≤99.82[A] |
| Test article for 1 minute | 4.75 | ≥1.75[A] | ≤3.00[A] | ≤99.90[A] |
| Test article for 5 minutes | 5.00 | ≥2.5[A] | ≤2.50[A] | ≤99.68[A] |
| Test article for 10 minutes | 5.00 | ≥2.75[A] | ≤2.25[A] | ≤99.44[A] |
| Positive control article | 4.83 | ≤1.50[B] | ≥3.33 | ≥99.95 |

[A] Toxicity observed but positive HA for virus obtained. The observed toxicity prevented the calculation of an absolute value.
[B] Limit of detection due to essential dilutions in the assay.

## 9.3 pH Measurements

The pH of GAMA Healthcare Clinell disinfectant was measured undiluted and at the 90% (v/v) dilution in MDCK infection media. The results shown below are for reference only.

***Table 3: pH of GAMA Healthcare Clinell Disinfectant***

| Conc % (v/v) | 100% | 90% |
|---|---|---|
| pH | 5.50 | 7.77 |

## 10 CONCLUSION

The tCPE observations of the toxicity assay indicate that the test article had some toxic effects on the MDCK cell line. These effects were observed at the $10^{-1}$ and $10^{-2}$ dilution level in all three wells of the serial dilution.

The cytotoxic effects interfered with recovery of virus at the $10^{-1}$ and $10^{-2}$ dilution level in some wells of the test article serial titration during virucidal assessment. This prevented the accurate determination of the $TCID_{50}$ of the test article incubated reactions.

The HA assay carried out during virucidal assessment was observed to recover virus at dilution levels below the level of toxicity, indicating the presence of virus at the levels of dilution below the observed cytopathic effect.

The HA results were used to determine a maximum level of virus reduction attributable to the test article; this was calculated at each of the different incubation time points. The observed cytotoxic effects were used to determine the minimum level of virus reduction attributable to the test article at each time point.

GAMA Healthcare Clinell Disinfectant reduced the viral titre by a potential maximum 3.00 - $\log_{10}$ $TCID_{50}$/ml (99.9%) at the 1 minute time point.

GAMA Healthcare Clinell Disinfectant reduced the viral titre of Avian Inluenza A NIBRG-14 by a minimum 1.25 - $\log_{10}$ $TCID_{50}$/ml (94.38%) and a maximum of 2.75 - $\log_{10}$ $TCID_{50}$/ml (99.82%) for the 30 second time point.

GAMA Healthcare Clinell Disinfectant reduced the viral titre of Avian Inluenza A NIBRG-14 by a minimum 1.25 - $\log_{10}$ $TCID_{50}$/ml (94.38%) and a maximum of 3.00 - $\log_{10}$ $TCID_{50}$/ml (99.90%) for the 1 minute time point.

GAMA Healthcare Clinell Disinfectant reduced the viral titre of Avian Inluenza A NIBRG-14 by a minimum 1.50 - $\log_{10}$ $TCID_{50}$/ml (96.84%) and a maximum of 2.50 - $\log_{10}$ $TCID_{50}$/ml (99.68%) for the 5 minute time point.

GAMA Healthcare Clinell Disinfectant reduced the viral titre of Avian Inluenza A NIBRG-14 by a minimum 1.50 - $\log_{10}$ $TCID_{50}$/ml (96.84%) and a maximum of 2.25 - $\log_{10}$ $TCID_{50}$/ml (99.44%) for the 10 minute time point.

## 11 ARCHIVE STATEMENT

The dedicated laboratory books, study protocol and final report, together with any other relevant information, may be held in Retroscreen Virology Ltd.'s secure archive for 12 months, from the issuing of the final report at no charge. After 12 months, storage may continue at the cost of £50.00 plus VAT per annum per box (W x D x H; 418mm x 710mm x 280mm) payable in advance or, at the discretion of the Sponsor, the material may be returned to the Sponsor.

### 11.1 Sample storage

All samples provided by the Sponsor and all samples generated during the research project will be disposed of three months after completion of the research project and the issue of the final report, unless otherwise requested by the Sponsor. Storage costs are £10.00 plus VAT per box (9 x 9 samples) per month. Retroscreen Virology Ltd reserves the exclusive rights on a small proportion of any samples recovered.

## 12 REFERENCES


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# 13 AMENDMENTS AND DEVIATIONS

## 13.1 Amendments

1) The study directorship of the study was transferred by management from Miss Kim Pham to Miss Elizabeth Moane, due to the absence of the former before the completion of the study.

## 13.2 Deviations

None.