CLB VIRUS SAFETY SERVICES

Final Report

FR3201

Process validation

"Lobator sd-1"

for inactivation of

BVDV

Applicant:	Telos Germany, co ltd. medical equipment Unter den Linden 26 6303 HUNGEN - OBBORNHOFEN GERMANY

Test facility:	Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Department Clinical Viro-Immunology Plesmanlaan 125 1066 CX Amsterdam The Netherlands

Code CLB:	*V*-21
Code applicant:	Lob-BVDV
Date initiation study:	April 2, 1996
Date completion study:	April 24, 1996
Report date:	May 8, 1996

RESPONSIBILITIES

Design, validation and performance of the disinfection procedures and femoral head measurement and temperature measurement were the responsibility of the applicant. CLB Virus Safety Services was responsible for virus preparation, virus titrations and biosafety aspects during performance of the study.

The project was carried out in a BSL3 (Biosafety on Level 3, CDC-NIH) classified laboratory at the Department of Clinical Viro-Immunology (Prof. F. Miedema, Head of the Department) at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service under direction of H. Schuitemaker, Ph.D (Project Manager of CLB Virus Safety Services).

Virus preparations and virus titrations were carried out at the laboratory of Clinical Viro-Immunology of the CLB by L. Berger, M.G. Holthuis, A.M. van der Hulst and E.A. Poelstra under direction of F.G. Terpstra (Manager Operations).

The study was subject to auditing by M.H. Post (Quality Assurance Manager of CLB Virus Safety Services).

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SUMMARY

The thermal disinfection process of Lobator sd-1 was tested for the ability to inactivate bovine viral diarrhoea virus (BVDV).

The effectiveness of this disinfection process was calculated by comparison of the amount of virus (cell-free + cell-associated) before treatment and the recovery of virus in the output sample and was expressed as the reduction factor.

After 62 minutes exposure to the thermal disinfection process of Lobator sd-1 (total process time is 92 minutes) no infectious virus was detectable.

The reduction factor (log_{10}) for the disinfection process was:

BVDV: $> 5.21^{*} \pm 0.25$

* Since the amount of inoculated virus was $10^{5.73}$ $TCID_{50}$ Units and the detection limit was $< 10^{0.52}$ $TCID_{50}$ Units, the reduction factor was > 5.21.

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1 *INTRODUCTION*

Transplantation of allogenic bone is a well accepted procedure all over the world. Allogenic transplants may be associated with the transmission of viruses, e.g. human immunodeficiency virus, hepatitis B and C virus, and parvovirus B19. The validation of procedures for viral inactivation would be an essential part in establishing the safety of bone transplants in lieu of serological screening tests. When efficient inactivation of relevant or model viruses by the procedure is demonstrated, this procedure is thought to be effective in inactivating any adventitious virus.

The objective of this study was to measure the efficacy of inactivation of bovine viral diarrhoea virus (BVDV) by applicant's thermal disinfection process of "Lobator sd-1". Because it belongs to the same virus family, BVDV is a specific model virus for hepatitis C virus. In addition BVDV is a general model virus for small enveloped RNA viruses.

The protocol of this study conforms to the requirements described in "Validation of virus removal and inactivation procedures" from the Commission of the European Communities (III/8115/89-EN, Final 1991) and the German Federal requirements "Requirements of validation studies for demonstrating the virus safety of medicinal products derived from human blood plasma" (Bundesanzeiger Nr. 84, Mai 1994).

2 *MATERIALS*

2.1 Test samples

The material (see appendix 1) used in the disinfection process was supplied by the applicant. The sample was delivered on the day of the experiment.

2.2 Reagents and equipment

Reagent and equipment supplied by the applicant and used in the disinfection process are listed in appendix 2 and 3.

2.3 Model viruses

Bovine viral diarrhoea virus (BVDV), strain NADL (VR-534, ATCC), an enveloped RNA virus (size: 40 nm).

The virus is kept as validated stock at CLB.

2.4 Cell lines

Embryonic bovine trachea (EBTr) cells (CCL-44, ATCC) are used for the preparation of the BVDV stock and BVDV infected cells, and for the titration assays.

The cell line is kept as validated cell bank at CLB.

2.5 Culture Media

EBTr cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% foetal calf serum (FCS), penicillin (100 IU/ml) and streptomycin (100 μg/ml). For preparation of a BVDV stock and for use in the $TCID_{50}$ assay, FCS was replaced by pooled human serum (HPS).

3 *METHODS*

All reagents and media used in the experiments were sterile. All equipment which was brought into direct contact with the materials had been sterilized.

3.1 Preparation of cell-free BVDV

Cell-free BVDV was prepared in EBTr cells. Uninfected cells were cultured in a tissue culture flask at 37°C. After 3-4 days, the monolayer of cells was inoculated with BVDV. The cells were observed for occurence of cytopathic effects (CPE) every day after

inoculation. The CPE typical for the used BVDV strain caused detachment of cells. When the majority of cells showed CPE, usually at day 4 or 5 after inoculation, the virus-cell suspension was collected. To remove cells and cell debris, the suspension was centrifuged for 15 min at 1000xg. The resulting supernatant was collected and stored at -70°C.

3.2 Preparation of cell-associated BVDV

At day 0 uninfected cells was cultured in a tissue culture flask at 37°C. At day 1, the monolayer of cells was inoculated with BVDV at high multiplicity of infection. At day 2 the infected EBTr cells were treated with trypsin/EDTA, collected and pelleted by centrifugation for 10 min at 350xg. The supernatant was removed and the cell pellet was resuspended in a small volume of culture medium. Finally the concentration of resuspended cells was determined.

3.3 Preparation and measurement of femoral heads

Femoral heads were prepared by the applicant; holes were drilled into the spongiosa of the femoral heads to allow insertion of a PCR tube, so that the PCR tube could be located in the center of the femoral head. Each femoral head was measured in two planes after all cartilage was removed. The first measurement (M1) in the coronal plane was taken from the most proximal point of the femoral head to the distal end of the femoral neck (transverse cut across the neck during removal of the femoral head from the patient). The second measurement (M2) in the transverse plane was taken across the diameter of the femoral head at its thickest part. This measurement was taken in several axes in the transverse plane and the minimum value was used.

3.4 Temperature measurement in the femoral heads

The temperature at the core of the femoral head was recorded during processing with the Lobator sd-1. A mobile programmable PC-supported measurement system with 4 channels, battery supported 32 kB memory and a V24/RS232 connector for on line data retrieval with a PC was used. A Ni-Cr-Ni insulated thermal electrode with a diameter of 0.5 mm and a length of 100 mm and a precision of 0.1°C was used as temperature sensor. The results of the temperature measurements were recorded on line on a PC with the support of a special IBM compatible software program. The results were stored as graphs and tables.
Prior to use the thermal electrode was calibrated in ice (0°C) and at room temperature (± 22°C).
The electrode was inserted into the center of the femoral head along side the PCR tube. Then the hole was sealed with a plastic lid and waterproof two component silicone glue so that no Ringer's lactate solution could leak through the hole into the bone.

3.5 Spiking of the femoral heads and performance of the inactivation process

Prior to use in the experiment, the frozen femoral head was thawed and adjusted to room temperature in a water bath and kept at this temperature for 2 hours.
A mixture of cell-free BVDV (batch 4) and cell-associated BVDV (final concentration of 10^6 cells/ml) in culture medium containing approximately 50% serum was prepared. A volume of 0.8 ml of this mixture was divided in two PCR tubes. The maximum volume of virus-

containing mixture that could be used in the study was limited by the size of the PCR tubes. In turn the maximum size of the PCR tubes used was limited by a concern to avoid removing an excessive amount of cancellous bone from the femoral heads. A sample of the mixture was titrated immediately and was labelled Lob-BVDV. One PCR tube was kept at room temperature for 62 minutes and was used as a bench control (label: Lob-BC-BVDV-62).
The second PCR tube and a thermal electrode were placed into the hole of the femoral head and thereafter parafilm was used to seal and cover the lid of the PCR tube. Onto the PCR tube a silicon glue was polymerized to prevent any access of liquid.
Then the femoral head was deposited into a polypropylene container filled with 250 ml of sterile Ringer's lactate. The heating program of the Lobator sd-1 was started with continuous stirring of the Ringer's lactate. After 62 minutes the femoral head was instantly taken out of the Ringer's lactate. The PCR tube was withdrawn from the femoral head and the content was titrated within 2.2 minutes after removal from the Ringer's lactate. For this purpose the 0.4 ml sample from the PCR tube was diluted in 24 ml of culture medium. From this mixture of 24.4 ml the dilutions as mentioned in Table 1 were made.
This sample was labelled Lob-BVDV-62*

* This sample was also tested in bulk cultures.

3.6 Control samples

For the calculation of the reduction factors, the viral titre as determined in the mixture of cell-free BVDV and cell-associated BVDV (Lob-BVDV) was used.

3.7 $TCID_{50}$ titration assays

BVDV $TCID_{50}$ values were determined using the BVDV-susceptible cell line EBTr. To each well of a 96-wells flatbottom microtitre plate 100 µl of an EBTr cell suspension (0.01×10^6 cells/ml in culture medium) were added. After 1 day of incubation at 37°C in a humidified incubator with 5% CO_2 the cells had adhered to the bottom of the wells. From the output sample a threefold serial dilution was made in culture medium. From each dilution step of the series, 8-plo 50 µl volumes were added to the wells with the adherent cells. The culture plates were again incubated at 37°C in a humidified incubator with 5% CO_2. After 6 days, the cultures were examined microscopically for the emergence of cytopathic effect (CPE) as reflected by detachment of the cells from the bottom of the culture well.

$TCID_{50}$ was defined as the reciprocal dilution that was able to infect 50% of the inoculated cultures under the conditions described above.

3.8 Testing for virus presence in a bulk system

Simultaneously and under the same conditions as the titration assay, a larger volume of the output sample was tested in two 175 cm^2 tissue culture flasks in a volume of 180 ml, for the presence of virus which might result in a lower detection limit. When no residual virus was found in the output samples when tested in the titration assay, the results of these culture flasks were used for the calculation of the viral titre and reduction factor.

4 *CALCULATION OF RESULTS*

The viral titre ($TCID_{50}$/ml) was calculated according to the formula:

$M = X_k + ½d - (d \times \text{Sum } p_i) + v$
where: M = log of the viral titre
X_k = log of the highest dilution that established infection in all replicates
d = log of the dilution factor
p_i = ratio of positive replicates/number of replicates, starting from the highest dilution that established infection in all replicates
v = log of the testvolume/well or flask (ml)

In case the lowest dilution did not establish infection in all replicates, the assumption was made that the dilution that theoretically would have come directly before the lowest dilution tested would have established infection in all replicates ($TCID_{50}$/ml $\leq$ calculated value).

In case the highest dilution still showed infection in one or more replicates, the assumption was made that the dilution that theoretically would have come directly after the highest dilution tested would have shown no infection ($TCID_{50}$/ml $\geq$ calculated value).

The standard error was calculated according to the formula:

$S_m = \pm \sqrt{[d^2 \times \text{Sum } \{p_i(1-p_i)/(n_i-1)\}]}$
where: S_m = standard error of the viral titre
d = log of the dilution factor
p_i = ratio of positive replicates/number of replicates, starting from the highest dilution that established infection in all replicates
n_i = number of replicates

The 95% confidence limit was calculated according to the formula:

95% CL = $S_m \times 1.96$
where: 95% CL = 95% confidence limit
S_m = standard error of the viral titre

In case of complete inactivation the viral titre (upper 95% confidence limit) was calculated according to the formula:

$TCID_{50}$/ml = log{(-ln 0.05)/total testvolume}

In case complete inactivation was found in the titration assay and incomplete inactivtion was found in the bulk culture, the minimal virus titre was calculated according to the formula:

$TCID_{50}$/ml $\geq$ log{1/testvolume}

The viral reduction factor was calculated according to the formula:

$$10^{rf} = \frac{\text{total infectious particles added}}{\text{total infectious particles recovered}}$$

The 95% confidence limit of the reduction factor was calculated according to the formula:

95% CL of reduction factor = $\sqrt{\{(95\%\ \text{CL before treatment})^2 + (95\%\ \text{CL after treatment})^2\}}$
where: 95% CL = 95% confidence limit

5 *CRITERIA*

5.1 A culture was considered positive if:

EBTr cells, growing in a confluent monolayer, showed CPE resulting in detachment of the cells from the bottom of the culture wells.

5.2 The assay was considered acceptable if:

At least 50% of the cultures of each dilution required for calculation of $TCID_{50}$ values could be interpreted.

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6 *RESULTS*

The number of positive cultures are shown in Table 1. Viral titres, standard errors and 95% confidence limits are shown in Table 2. Reduction factors (rf) and their 95% confidence limits are shown in Table 3.
The results of the temperature measurements are shown in Appendix 4. The results of the femoral head measurements and the Lobator sd-1 identification are shown in Appendix 5.

Table 1: Number of positive cultures out of a total of 8 replicates per dilution step*

Lob-BVDV												
Dilution 3^{-}	3	4	5	6	7	8	9	10	11	12	13	14
Positive cultures	8	8	7	6	2	0	0	0	0	0	0	0
Lob-BVDV-62												
Dilution 3^{-}	0	1	2	3	4	5	6	7	8	9	10	11
Positive cultures		0	0	0	0	0	0	0	0	0	0	0
Lob-BC-BVDV-62												
Dilution 3^{-}	3	4	5	6	7	8	9	10	11	12	13	14
Positive cultures	8	8	8	2	2	2	0	0	0	0	0	0

☐ = not applicable

* Unless indicated otherwise

Table 2: Calculated values

Sample	$TCID_{50}$/ml	Standard error	95% Confidence limit
Lob-BVDV (virus/cell-mixture)	$10^{6.13}$	0.13	0.25
Lob 6-BVDV-62[1]	$< 10^{3.14}$		
Lob 6-BVDV-62^	$< 10^{0.92}$		
Lob-BC-BVDV-62 (bench control)	$10^{6.07}$	0.14	0.27

☐ = not applicable

[1] Calculation of the $TCID_{50}$/ml: $\log\{(\ln 0.05)/(0.4/183)\}$.
^ A volume of two times 11 ml was tested in a bulk culture and no infectious virus was detectable. Calculation of the $TCID_{50}$/ml: $\log\{(\ln 0.05)/(22/61)\}$.

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Table 3: Viral reduction factors

Sample	Reduction of virus						
	Virus recovered $TCID_{50}$ Units/ml	Volume of sample (ml)	Fraction of virus inoculum*	Virus inoculated $TCID_{50}$ Units	Total $TCID_{50}$ Units recovered	Reduction factor	95% Confidence limit
Lob-BVDV (virus/cell-mixture)	$10^{6.13}$	0.4			$10^{5.73}$		0.25
Lob 6-BVDV-62	$< 10^{3.14}$	0.4	1.0	$10^{5.73}$	$< 10^{2.74}$	>2.99	0.25
Lob 6-BVDV-62^	$< 10^{0.92}$	0.4	1.0	$10^{5.73}$	$< 10^{0.52}$	>5.21	0.25
Lob-BC-BVDV-62 (bench control)	$10^{6.07}$	0.4	1.0	$10^{5.73}$	$10^{5.67}$	0.06	0.37

☐ = not applicable

* Ratio of volume of virus inoculum present in sample and volume of virus inoculated in starting material.
^ A volume of two times 11 ml was tested in a bulk culture and no infectious virus was detectable.

7 *CONCLUSIONS*

The study was valid; the criteria were met.

After 62 minutes exposure to the thermal disinfection process of Lobator sd-1 (total process time is 92 minutes) no infectious virus was detectable.

The reduction factor (log_{10}) for the disinfection process was:

BVDV: $> 5.21^{*} \pm 0.25$

* Since the amount of inoculated virus was $10^{5.73}$ $TCID_{50}$ Units and the detection limit was $< 10^{0.52}$ $TCID_{50}$ Units, the reduction factor was > 5.21.

8 *RECORD MAINTENANCE*

Protocol, modifications, a copy of the reports and all correspondence between CLB Virus Safety Services and the applicant will be maintained within a file in the CLB. These records will be retained for a period of ten years following submission of the final report to the applicant.

9 *APPROVALS*

11/06/96

F.G. Terpstra
Manager Operations

Date

11 06 96

Dr. H. Schuitemaker
Project Manager

Date

10 *QUALITY ASSURANCE STATEMENT*

This final report has been audited by the Quality Assurance Manager of CLB Virus Safety Services and has been found to describe the methods used and to reflect the raw data of the study.

11 06 96

M.H. Post
Quality Assurance Manager

Date

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Appendix 1: List of samples obtained from applicant

Starting materials

Sample name	Amount of sample obtained	Sample designation
Femoral head	1 piece	Lob-BVDV-62 (V-21B)

Appendix 2: Reagents supplied by applicant

- Ringer's lactate (sterile)

Appendix 3: Equipment supplied by applicant

- Lobator sd-1 (serial number 1090)
- PC with IBM compatible software (Online 2, version 4.32, DEMA Software GmbH)
- PCR tubes with caps (size: 0.45 ml)
- Silicon glue, two component
- Sterile disinfection container, complete
- Temperature sensors, four Ni-Cr-Ni insulated thermal electrodes with a diameter of 0.5 mm and a length of 100 mm (T430, Ahlborn GmbH, Holzkirchen, Germany), precision 0.1°C
- Therm 2281-8, Ahlborn Mess- und Regelungstechnik GmbH, Holzkirchen, Germany with 4 channels, battery supported 32 kB memory and a V24/RS232 connector

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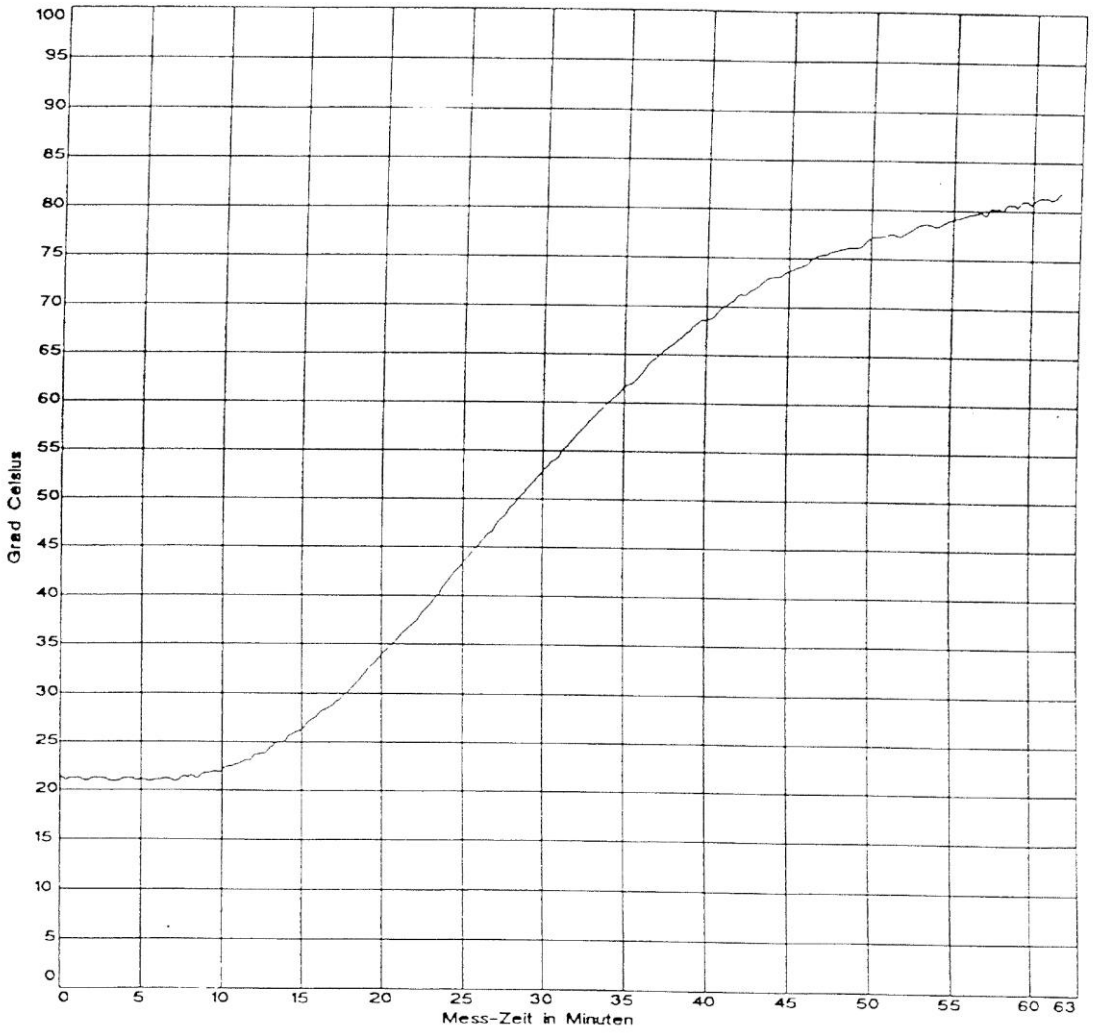
Appendix 4: Temperature measurement of Lob-BVDV-62



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Appendix 5: Measurement of femoral heads

Femoral head measurements and Lobator sd-1 identification

Sample	M1 (mm)	M2 (mm)	Lobator sd-1 (serial number)
Lob-BVDV-62	54	52	1090

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BVDV inactivation in bone by Lobator sd-1 treatment