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Biologicals 33 (2005) 95–99
BIOLOGICALS
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SARS-coronavirus (SARS-CoV) and the safety of a solvent/detergent (S/D) treated immunoglobulin preparation

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Received 6 July 2004; accepted 12 January 2005

Abstract

SARS-coronavirus (SARS-CoV) is a newly emerged, highly pathogenic agent that caused over 8000 human infections with nearly 800 deaths between November 2002 and September 2003. While direct person-to-person transmission via respiratory droplets accounted for most cases, other modes have not been ruled out. SARS-CoV viraemia does not seem to reach high titres, however, it has to be excluded that virus transmission may occur via blood transfusion or application of therapeutic plasma products, e.g. fresh-frozen plasma or single components derived thereof. Manufacturing processes of all plasma derivatives are required to comprise dedicated virus inactivation/removal steps. Treatment with a mixture of solvent and detergent (SD) has successfully been applied to inactivate the most members of the transfusion-relevant viruses without affecting therapeutic properties of the products. The SD treatment irreversibly disrupts the lipid envelope of viruses such as HIV, HBV, HCV, HGV and CMV. In this study we evaluated the manufacturing process of an immunoglobulin preparation (OCTAGAM, manufactured by Octapharma Pharmazeutika Produktionsges.m.b.H., Vienna, Austria) for its capacity to inactivate the SARS-CoV. Our results demonstrate that SARS-CoV was completely inactivated below the limit of detection. This was found to occur within 1 min of SD treatment.

1. Introduction

The Severe Acute Respiratory Syndrome (SARS) is a novel infectious disease that first occurred in China in November 2002, but was not known to the public before February 2003. SARS is caused by a newly emerged virus belonging to the coronaviridae family, provisionally termed SARS-coronavirus (SARS-CoV) [8]. Available sequence data indicate that SARS-CoV is clearly different from all previously known coronaviruses [20]. A new, fourth genetic lineage has been proposed for SARS-CoV [15].

Although human-to-human transmission of SARS-CoV is less efficient than for other air-borne viruses such as influenza A viruses, the recent SARS epidemic was characterised by several explosive outbreaks [4,24]. Most infections resulted from direct transmission via respiratory droplets during close personal contact and adequate protective measures were shown to be effective [22,25]. However, there are a number of instances when transmission occurred through other modes not yet well defined. In central China, around 50% of probable SARS patients did not have an apparent history of close personal contact with another victim [14]. At least a portion of these cases may have arisen from modes of transmission different from respiratory droplets. Besides virus shedding into stool samples, SARS-CoV viraemia occurs, but does not seem to reach high titres (≈200 copies/ml) [16,21]. During the SARS-CoV-epidemic,

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1045-1056/05/$30.00
doi:10.1016/j.biologicals.2005.01.003

concerns were raised about the possibility of SARS-CoV transmission by transfusion of blood components or therapeutic plasma products. Manufacturing processes of all plasma derivatives are required to comprise dedicated virus inactivation/removal steps for enveloped viruses [5]. Treatment with a mixture of solvent and detergent (SD) is used by many manufacturers worldwide and has successfully been applied to clotting factor and immunoglobulin preparations [2,11,17]. The SD treatment causes enveloped viruses to be irreversibly destroyed. These include the human immunodeficiency virus types 1 + 2 (HIV-1, HIV-2), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis G virus (HGV) and human cytomegalovirus (CMV) [17].

On the basis of existing validation data for enveloped viruses, it can be anticipated that the inactivation/removal steps incorporated into manufacturing processes for plasma-derived medicinal products will also be effective for SARS-CoV. To confirm this assumption, verification studies with SARS-CoV were performed for the SD treatment conditions used in the manufacturing of an immunoglobulin concentrate intended for intravenous application (OCTAGAM, manufactured by Octapharma Pharmazeutika Produktionsges. m.b.H., Vienna, Austria). In full-scale manufacturing, SD process conditions include treatment with 0.3% (w/w) tri(*n*-butyl)phosphate (TNBP) and 1.0% (w/w) Octoxynol (trade name: Triton X-100) at 6.0 ± 0.5 °C and at pH 5.3 ± 0.2 for a minimum of 4 h. In compliance with the CPMP [5,6], the robustness of each virus inactivation method has to be demonstrated experimentally. Therefore, the inactivation of SARS-CoV by SD treatment was investigated in the laboratory scale at lowered concentration of solvent and detergent (75% of standard SD concentration) and at a shortened process time (30 min).

2. Materials and methods

2.1. Viruses and cells

The SARS-CoV isolate FFM-1 [7] was obtained from the sputum of a patient hospitalised with a diagnosis of probable SARS in the Isolation Unit of Frankfurt University Hospital, Germany. Virus stocks were stored at −80 °C. The maintenance medium consisted of minimum essential medium (MEM) without foetal calf serum (FCS) and was supplemented with 100 IU/ml of penicillin and 100 μg/ml of streptomycin. Virus was propagated and titrated on confluent Vero cells and titres were determined as 50% tissue culture infective doses ($TCID_{50}$/ml) in 96-well microtitre plates with 8 replicates per dilution (10-fold) and 50 μl inoculum per well [12,23]. The initial titre of the undiluted virus stock was 1.12×10^7 $TCID_{50}$/ml (= 7.05 ± 0.35 $\log_{10}$ $TCID_{50}$/ml [hereafter referred to as virus stock control 0 min]) and 2×10^7 $TCID_{50}$/ml (= 7.30 ± 0.44 $\log_{10}$ $TCID_{50}$/ml [virus stock control 30 min]).

In accordance with WHO recommendations, all work involving infectious SARS-CoV was performed under bio-safety level (BSL)-3 conditions in a BSL-3 facility.

2.2. Study design

The validation experiments were performed according to current CPMP guidelines and the recommendations of the Paul-Ehrlich-Institut in Germany [3,5,6]. The aim of the study was to evaluate the total capacity of the SD process for inactivating SARS-CoV and to define the minimal incubation time to inactivate the viruses below the detection limit. The exact incubation times with SD were 1, 3, 5, 10, 20 and 30 min (Fig. 1). Furthermore, the purpose of this validation study was to investigate the impact of a significantly lowered concentration of TNBP and Octoxynol (75% of standard SD concentration, i.e. 0.23% (w/w) TNBP/0.75% (w/w) Octoxynol) on virus inactivation. SD treatment was performed at 6.0 ± 0.5 °C and at pH 5.3 ± 0.2 for the above mentioned incubation periods in two independent tests.

A down-scaled version of the manufacturing process examined was established and the equivalence of the down-scale and manufacturing-scale intermediate was demonstrated, e.g. by measurement of their protein concentrations.

For the purification of IgG from cryopoor plasma, the Cohn–Oncley method is applied. This process involves

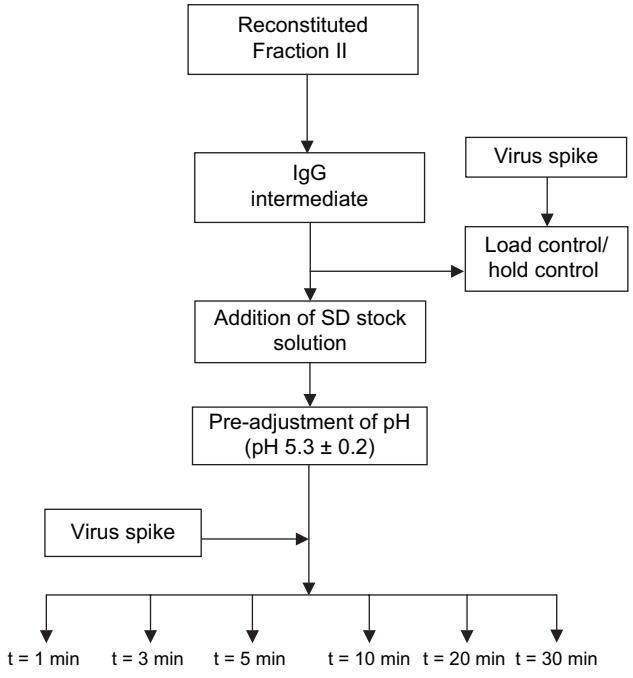


Fig. 1. Impact of a SD process on inactivation of SARS-CoV in an immunoglobulin preparation (OCTAGAM).

the sequential purification of protein fractions by adding various concentrations of ethanol with concurrent adjustments in pH, ionic strength and temperature. The precipitation and separation of fraction I + III from fraction II is considered the most viral safety relevant step for the non-lipid enveloped viruses. Starting materials in this study were process intermediates (resuspended fraction II) obtained from the manufacturing scale, which were spiked 1 in 20 with virus. An aliquot of the starting material without SD was individually spiked also 1 in 20 with virus and titrated to confirm the amount of virus added. This spiked intermediate (without SD) was titrated at the beginning of the process (virus-spiked intermediate control 0 min) and at the end of the process (virus-spiked intermediate control 30 min). This separate spiking was required, because, in the presence of SD reagents, the initial titre of any virus-spiked process intermediate would not represent the quantity of virus actually inoculated. Infectivity was determined by endpoint titration on Vero cells. The SD treatment was terminated in the laboratory studies by diluting 1:250 with cell culture medium. In order to prove that the inactivation by SD was terminated by our dilution procedure, an aliquot (100 µl) of the SD containing intermediate was prediluted 1:237.5 with cell culture medium. This solution was then spiked with virus (1 in 20), resulting in a final dilution factor of 1:250 with respect to SD (termination control). Specific non-spiked process intermediates were obtained for control procedures and tested for their potential cytotoxicity (see below) for the Vero cells and for their potential interference with the detection of low virus titres. All controls were serially 10-fold diluted and eight cultures (inoculum size 0.05 ml) of each dilution were established.

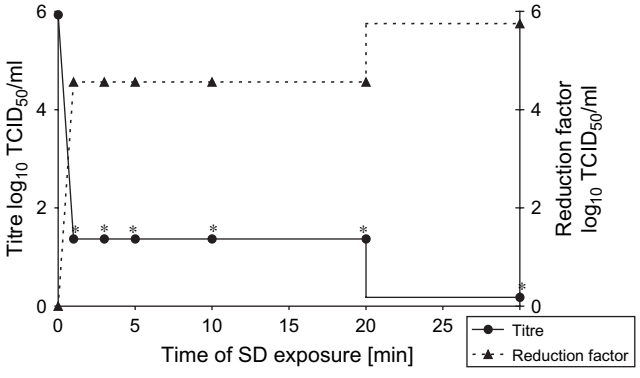
From the samples taken after 1, 3, 5, 10 or 20 min of SD treatment, 48 × 1 ml of the 1:250 dilution (corresponds to 192 µl of undiluted sample) was used as inoculum, but for 30 min the inoculum volume was increased to 720 × 1 ml (corresponds to 2.88 ml of undiluted sample). The cell cultures were maintained in a CO_2 incubator at 37 °C. Vero cultures were examined microscopically and evaluated for virus-induced (or cytotoxic) cell changes. The virus titres were calculated according to the method of Spearman and Kärber [12,23]. The virus titre for samples where no virus replication is observed, can be determined by using the Poisson distribution at 95% upper confidence limits. For the conversion to $TCID_{50}$/ml, the concentration of virus particles is divided by ln 2. Therefore, if virus replication was below the limit of detection, a theoretical, though very low content of viruses had to be accepted (1.37 $\log_{10}$ $TCID_{50}$/ml for 1, 3, 5, 10, 20 min and 0.18 $\log_{10}$ $TCID_{50}$/ml for 30 min process time). The virus reduction factor, which quantitatively determines the capacity of the process step to inactivate viruses, was calculated from the ratio of the virus titre detectable in spiked OCTAGAM intermediate without SD and the virus titre potentially present after the SD step.

3. Results

For the study, appropriate in-process samples were collected from OCTAGAM full-scale production. Before pre-adjustment of the pH, this process intermediate was supplemented with SD and finally spiked with SARS-CoV at a ratio of 1 in 20. This starting material (with only 0.23% [w/w] TNBP and 0.75% [w/w] Octoxynol) was incubated at 6.0 ± 0.5 °C for 30 min. To determine the kinetics of virus inactivation, test samples were taken at predefined intervals during the process (1, 3, 5, 10, 20 and 30 min). The SD treatment was terminated by a 1:250 dilution with cell culture medium. The diluted test samples were screened with susceptible cells (Vero) for SARS-CoV infectivity by bulk analysis.

The SARS-CoV titre detected in the starting material prior to SD treatment was 5.93 ± 0.25 $\log_{10}$ $TCID_{50}$/ml. The results of Fig. 2 and Table 1 demonstrate that SARS-CoV was completely inactivated below the limit of detection within 1 min of SD treatment. Depending on the sample volume tested the detection limit varied between 1.37 $\log_{10}$ $TCID_{50}$/ml and 0.18 $\log_{10}$ $TCID_{50}$/ml. In both independently performed tests similar results were obtained. Taking into consideration the various handling procedures during the laboratory experiment (e.g. spike with virus, careful mixing of the virus-spiked intermediate, sterile sampling of an aliquot, dilution with cell culture medium for terminating the inactivation process), less than 1 min was required. The resulting log reduction factor is ≥5.75 ± 0.25 $\log_{10}$ $TCID_{50}$/ml.

Both virus stock controls and virus-spiked intermediate controls showed the expected values and demonstrated the validity of the study (Table 1). This means that the differences of the virus titre at the beginning and at the end of the incubation period was <0.5 $\log_{10}$



* infectivity was below the limit of detection

Fig. 2. SARS-CoV inactivation by SD treatment – influence of incubation time.

Table 1
SARS-CoV inactivation by SD treatment

Sample description	Dilution of sample/culture volume/test sample volume	Positive cultures	Virus titre $\log_{10}$ $TCID_{50}$/ml	Reduction factor $\log_{10}$
Virus stock control 0 min	–	–	7.05 ± 0.35	–
Virus stock control 30 min	–	–	7.30 ± 0.44	–
Virus-spiked intermediate control 0 min	–	–	5.93 ± 0.25	–
Virus-spiked intermediate control 30 min	–	–	6.30 ± 0.44	–
Termination control	–	–	5.93 ± 0.25	–
1 min SD exposure	$1:250/2 \times 24 \times 1$ ml/0.192 ml	0/48	≤ 1.37	$\geq 4.56 \pm 0.25$
3 min SD exposure	$1:250/2 \times 24 \times 1$ ml/0.192 ml	0/48	≤ 1.37	$\geq 4.56 \pm 0.25$
5 min SD exposure	$1:250/2 \times 24 \times 1$ ml/0.192 ml	0/48	≤ 1.37	$\geq 4.56 \pm 0.25$
10 min SD exposure	$1:250/2 \times 24 \times 1$ ml/0.192 ml	0/48	≤ 1.37	$\geq 4.56 \pm 0.25$
20 min SD exposure	$1:250/2 \times 24 \times 1$ ml/0.192 ml	0/48	≤ 1.37	$\geq 4.56 \pm 0.25$
30 min SD exposure	$1:250/30 \times 24 \times 1$ ml/2.88 ml	0/720	≤ 0.18	$\geq 5.75 \pm 0.25$

Virus stock control: virus titre of the undiluted virus stock at 0 min and 30 min process time. Virus-spiked intermediate control: titre of a 1 in 20 virus-spiked intermediate (without SD) at 0 min (represents the initial virus titre for calculating the reduction factors) and 30 min process time. Termination control: virus spiked 1 in 20 into process intermediate with 1:250 diluted SD.

$TCID_{50}$/ml. Furthermore, the termination control confirmed that the 1:250 dilutions were more than sufficient to terminate the inactivation of SARS-CoV by SD (titre difference $<0.5 \log_{10}$ $TCID_{50}$/ml). In a cytotoxicity and interference pre-study, this dilution was found to be non-cytotoxic and did not interfere with the SARS-CoV/Vero cells system (titre difference $<0.5 \log_{10}$ $TCID_{50}$/ml).

4. Discussion

The SARS outbreak seems to have been halted for the present time, indicating that the stringent control measures taken to prevent person-to-person transmission were effective. Nevertheless, it remains important to assess the possible risk for other modes of spread, for example via blood transfusion or application of therapeutic plasma products such as immunoglobulins. In general, the level and duration of viraemia and viral loads in plasma pools are critical parameters for risk assessment from emerging viruses. The risk of SARS-CoV transmission via blood or therapeutic plasma products seems remote, particularly because SARS-CoV viraemia does not seem to reach long-lasting high titres [16,21]. In order to reduce the potential risk of SARS-CoV transmission by transfusion of cellular blood components, screening of blood donors by nucleic acid techniques (NAT), e.g. PCR, might be useful [21]. Furthermore, it is valuable to obtain information about the anti-SARS-CoV efficacy of virus inactivation steps like the SD procedure, which is widely used by many companies world-wide in the manufacture of therapeutic plasma-derived products including immunoglobulins. The acceptance of such pharmaceutical products does not rely on their therapeutic benefit alone, but to a large extent on their documented viral safety [13]. The SD method irreversibly disrupts the lipid coat of enveloped viruses and its associated binding sites under mild process conditions. The efficacy of the SD method has been extensively validated with human and model viruses, as well as by animal studies under both standard and robustness conditions of the product-specific manufacturing processes [1,9,17]. Due to the non-selective mode of action, the SD method is capable of inactivating not only the viruses of most concern, e.g. HIV, HBV and HCV, but also those viruses not tested for during routine screening at the blood collection centres, e.g. human T-lymphotropic virus (HTLV)-1 + 2, HGV and CMV.

The safety margins of plasma-derived medicinal products are validated utilising down-scaled models of their respective manufacturing processes in order to investigate the inactivation capacity of a broad range of physicochemically diverse model viruses. In the case of SARS-CoV, several facts argued against its relevance in terms of plasma product safety. Firstly, as already mentioned, the reported SARS-CoV titres in plasma are low. Secondly, the extensive number of validation studies using other enveloped viruses indicates that any other enveloped virus, such as SARS-CoV, would become properly inactivated as well. On the other hand it could be shown that SARS-CoV has some differences in behaviour towards physicochemically noxa in comparison to other known human coronaviruses [18], while it is easily destroyed by common disinfectants [19]. Thus, it is prudent to perform validation studies in order to confirm the safety hypothesis.

In our investigation, the SD treatment of OCTAGAM process intermediates resulted in a very rapid inactivation to below the detection limit of the SARS-CoV. Compared to routine manufacturing of OCTAGAM, the

conditions under which SD treatment was performed were challenged significantly. The inactivation time was reduced by more than 95%, whereas the concentration of SD reagents was decreased to only 75% of production conditions. Despite the worsened process conditions and the improved level of detection, infectious SARS-CoV could not be found after 1 min of SD treatment, demonstrating the robustness of virus inactivation by this technology.

Taken into account the starting virus titre, the reduction factor was $\geq 5.75 \pm 0.25 \log_{10} TCID_{50}$/ml. Our current investigation confirmed the predicted behaviour of SARS-CoV, when treated with the SD under the described conditions, and corroborated earlier findings [2,10]. This is not surprising, as the SD method confirmed to be an effective technology used for the inactivation of enveloped viruses [2].

In conclusion, the results obtained in our investigation demonstrated that the process conditions specified for the SD treatment of OCTAGAM are very sufficient to inactivate enveloped viruses such as SARS-CoV to below the limit of detection. The safety margin is very high, as the detection limit was already reached within 1 min of SD exposure even at a significantly reduced concentration of TNBP and Octoxynol. Furthermore, with such a convincing inactivation capacity and kinetic at extreme process conditions, the efficacy of the standard SD treatment used for OCTAGAM to inactivate SARS-CoV must be even higher than the one demonstrated by our study, even if the virus load were higher than the one simulated in this study.

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