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Optimization of the inactivation process of porcine circovirus 2 by binary ethyleneimine

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Abstract
Complete inactivation of porcine circovirus 2 (PCV2) at 37 °C using binary ethylene imine (BEI) at a final concentration of 0.1, 0.25, 0.5, 1.0, 1.5 and 3.0 mM was observed at 14, 14, 12, 10, 10 and 8 hours respectively, with inactivation rate ranging from 0.53 to 1.05 $\log_{10}$/hour. At 25 °C the complete PCV2 inactivation was observed at 28, 28, 24, 24, 20 and 12 hours using BEI at a final concentration 0.1, 0.25, 0.5, 1.0, 1.5 and 3.0 mM, respectively, with inactivation rate ranging between 0.2 - 0.79 $\log_{10}$/hour. The "total inactivation time" for completely inactivating the infectivity of 100 mL of $10^{6.3}$ $TCID_{50}$/mL PCV2 with 3 mM BEI at 25 °C was determined as 36 hours.

Keywords: Porcine circovirus 2, binary ethylene imine, virus inactivation

Introduction
Porcine circovirus 2 (PCV2) is an economically important viral pathogen, which causes conditions such as postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), and reproductive failure in pigs [1]. PCV2 is a small (17 nm in diameter), non-enveloped virus with a circular DNA genome of around 1.7 kb. The capsid protein of PCV2, comprising 233 or 234 amino acid residues, is the only structural protein. It belongs to the genus Circovirus of the family Circoviridae. PCV2 infects many types of cells in pigs, such as lymphocytes, macrophages, hepatocytes, and cardiomyocytes [2]. PCV2 replicates in the nucleus of infected cells, using the host polymerase for genome amplification1. PCV2 infection can cause immunosuppression, inflammation, and apoptosis in pigs [1, 2]. PCV2 is transmitted through body secretions, such as saliva, nasal discharge, urine, feces, and semen. PCV2 can also be transmitted vertically from sow to fetus. The clinical signs of PCV2 infection depend on the level of viremia in the infected pigs. Some common signs are weight loss, wasting, respiratory distress, enlarged lymph nodes, skin lesions, kidney damage, and reproductive problems, such as early embryonic death, abortions, still birth and neonatal mortality [1, 2]. Vaccination against PCV2 has been shown to reduce the incidence and severity of disease, as well as improve growth performance and survival rate of pigs [1]. Currently, inactivated vaccines and subunit vaccines incorporating the recombinant capsid protein are commercially available [1, 2]. Virus inactivation and safety tests are the most critical steps in the production of inactivated vaccines.
Historically, formalin was a commonly employed chemical to inactivate microbes for vaccine preparation. However, formalin was known to degrade the structure of the virion and the antigenicity of virus proteins, if used at higher concentration or for longer time [3, 4]. On the other hand lower concentration or duration of inactivation with formalin may lead to persistence of the infective virus. Formalin does not produce a first order kinetic reaction when inactivating the virus [4, 5]. Later, aziridine group inactivators like Acetylethyleneiemine (AEI) [5, 6] were preferred compared to formaldehyde treatment for inactivation. In 1974, Bahnemann [7, 8], reported use of 2-bromoethyleneamine (BEA) for inactivation of Foot and Mouth disease virus, in alkaline solution, which is called binary ethyleneimine (BEI), to reduce the toxicity of ethyeneimine (EI). The BEI method developed by Bahnemann [7, 8, 9] circumvents the direct handling of the very toxic other aziridine and has become the most popular agent for inactivation of viral antigens for veterinary vaccines [9]. The present study determined the optimal inactivation concentration and time of inactivation of PCV2 using BEI as inactivating agent to arrive at the total inactivation time [10] for vaccine production.

Materials and Methods

Cell culture

PK-15 cells, obtained from National Centre for Cell Sciences, Pune, India, were maintained at 37 °C under 5% CO_2 concentration in Minimum essential media (Invitrogen), pH 7.2, supplemented with 5% Foetal Bovine Serum (Sigma, USA) and antibiotic-antimycotic solution (Invitrogen).

PCV2 virus and propagation

The PCV2 virus used in this study was isolated from a pig farm in Tirupur, Tamil Nadu, India, during 2013. The partial gene sequence of the capsid protein was accessioned in the Genbank (KJ784655). The virus propagation was performed in PK-15 cells cultured in sterile plastic cell culture flasks as described above.

Virus titration

The end point titration of the PCV2 containing samples were performed in 80% confluent PK-15 cells cultured in sterile 96 well plastic cell culture plates (Invitrogen). 10-fold serially diluted test samples were used for infecting PK-15 cells and 72 hours post infection, the infected cells were fixed in 2% paraformaldehyde. The fixed cells were permeablilized with 0.1% Triton-X100 in phosphate buffered saline for immunofluorescence assay. Briefly, the infected cells were incubated with anti-PCV2 serum from pigs followed by staining with FITC conjugated secondary antibody and examined under fluorescent microscope. The $TCID_{50}$ was determined using the Reed–Muench method.

Chemicals

2-Bromoethylamine hydrobromide (BEA), sodium hydroxide (NaOH), sodium thiosulphate ($Na_2S_2O_3$. $5H_2O$) were obtained from Sigma. The BEA was used to prepare binary ethyleneimine (BEI). Briefly, 0.175N NaoH was prepared in sterile distilled water and the BEA was dissolved in the 0.175N NaOH solution to make a final concentration of 0.1M BEA. The solution was incubated at 37 °C for 15 minutes at 37 °C to facilitate the conversion of BEA to BEI.

Inactivation of PCV2

The inactivation of PCV2 was carried out under sterile conditions at 37 °C or 25 °C with 0.1, 0.25, 0.5, 1.0, 1.5 and 3.0 mM of BEI. A volume of 100 mL of PCV2 virus was taken for inactivation experiment with each concentration of BEI. Samples were collected under sterile precautions at 2 or 4 hours intervals in a biosafety cabinet for estimation of the infectious PCV2 titre. The titre was estimated by immunofluorescence assay. At the end of the inactivation experiment 1 M sodium thiosulfate solution was added at the rate of $1/10^{th}$ of the volume of BEI used for inactivation and incubated for 30 minutes to neutralize BEI activity. Samples collected at different time points of initiation of inactivation process were also neutralized with 1 M sodium thiosulfate solution.

Results

The complete loss of infectivity of PCV2 at 37ºC, with no residual virus, was achieved at 14, 14, 12, 10, 10 and 8 hours after the start of inactivation process with BEI of final concentrations, 0.1, 0.25, 0.5, 1.0, 1.5 and 3 mM, respectively (Fig. 1, Table 1). The inactivation rate ranged from 0.53 to 1.05 $log_{10}TCID_{50}$/hour (Table 3).

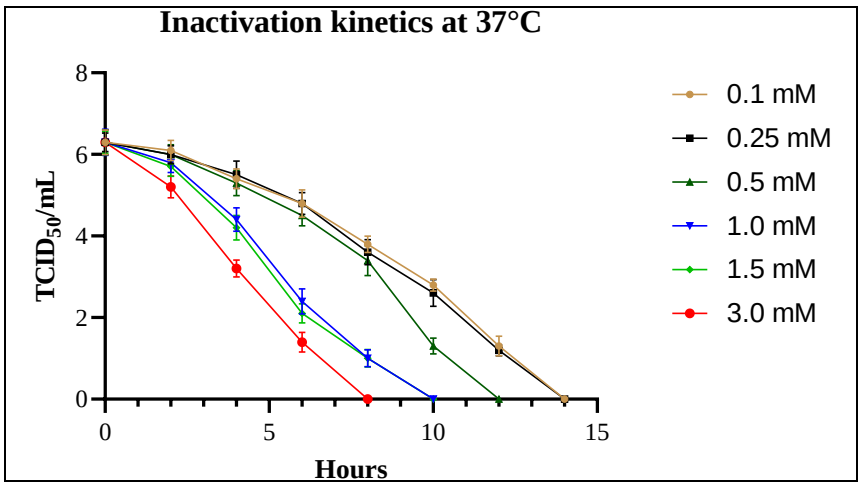

Fig 1: Inactivation of PCV2 virus with BEI at 37 ºC

Table 1: Inactivation of PCV2 virus with BEI at 37 °C

Titer expressed in log10						
Hours of inactivation	**Mean virus titer (log10 TCID50/ml) with different BEI molarities**					
	0.1 mM	**0.25 mM**	**0.5 mM**	**1.0 mM**	**1.5 mM**	**3.0 mM**
0	6.3	6.3	6.3	6.3	6.3	6.3
2	6.1	6.0	6.0	5.8	5.7	5.2
4	5.4	5.5	5.3	4.4	4.2	3.2
6	4.8	4.8	4.5	2.4	2.1	1.4
8	3.8	3.6	3.4	1	1	0
10	2.8	2.6	1.3	0	0	0
12	1.3	1.2	0	0	0	0
14	0	0	0	0	0	0

The complete loss of infectivity of PCV2 at 25 °C, with no residual virus, was achieved at 28, 28, 24, 24, 20 and 12 hours after the start of inactivation process with BEI of final concentrations, 0.1, 0.25, 0.5, 1.0, 1.5 and 3 mM, respectively (Fig.2, Table 2). The inactivation rate ranged from 0.20 to 0.79 $log_{10}TCID_{50}$/hour (Table 3).

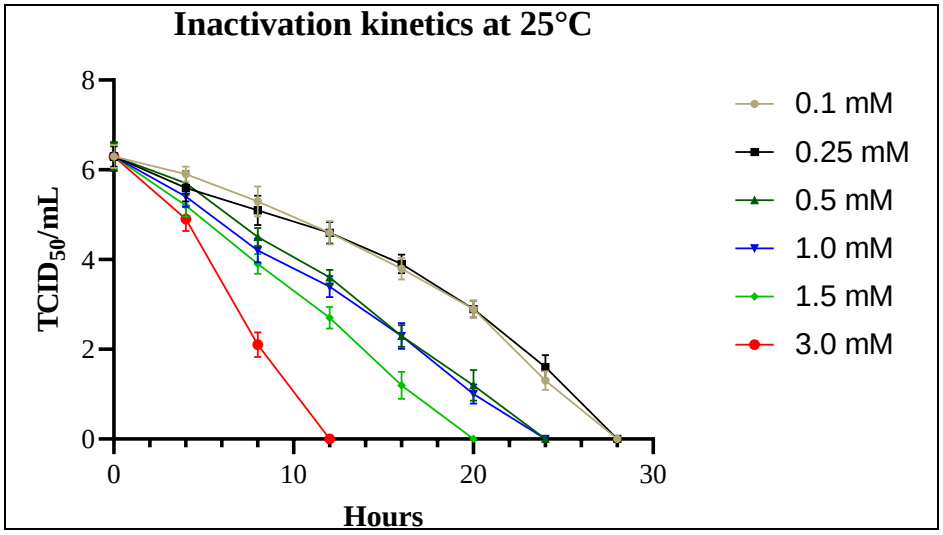

Fig 2: Inactivation of PCV2 virus with BEI at 25 °C

Table 2: Inactivation of PCV2 virus with BEI at 25 °C

Titer expressed in log10						
Hours of inactivation	**Mean virus titer (log10 TCID50/ml) with different BEI molarities**					
	0.1 mM	**0.25 mM**	**0.5 mM**	**1.0 mM**	**1.5 mM**	**3.0 mM**
0	6.3	6.3	6.3	6.3	6.3	6.3
4	5.9	5.6	5.7	5.4	5.2	4.9
8	5.3	5.1	4.5	4.2	3.9	2.1
12	4.6	4.6	3.6	3.4	2.7	0
16	3.8	3.9	2.3	2.3	1.2	0
20	2.9	2.9	1.2	1.0	0	0
24	1.3	1.6	0	0	0	0
28	0	0	0	0	0	0

Table 3: Inactivation rate (log10/hour) with BEI (0.1 and 1.5 mM)

Temperatures	**Virus inactivation rate with different BEI molarities**					
	0.1 mM	**0.25 mM**	**0.5 mM**	**1.0 mM**	**1.5 mM**	**3.0 mM**
37ºC	0.53	0.53	0.63	0.79	0.79	1.05
25ºC	0.20	0.20	0.22	0.26	0.39	0.79

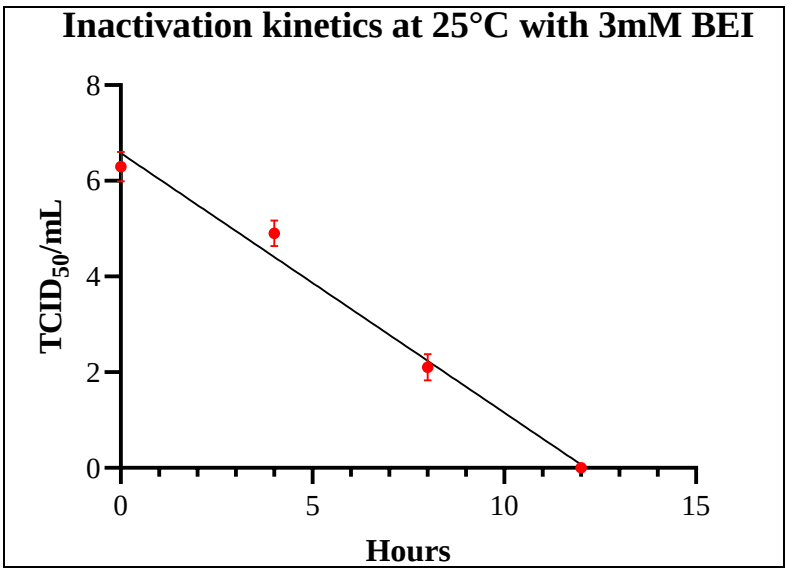

Fig 3: Inactivation rate (log10/hour) with BEI (0.1 and 1.5 mM)

Under the study conditions and at 25 °C, the preferred temperature for inactivation of viruses without degradation of the antigens by thermal inactivation, the complete loss of infectivity is achieved at 12 hours (Fig. 3). The inactivation rate of PCV2 with 3 mM final concentration of BEI at 25 °C was 0.79 $\log_{10}TCID_{50}$/hour.

Discussion

Porcine circovirus 2 is a major pig pathogen around the world. The first inactivated vaccines for PCV2 were available in 2004 in Europe and in 2006 in North America [1]. This was followed by development and commercial sale of baculovirus expressed recombinant PCV2 capsid protein based vaccines from 2008. Six genotypes of PCV2, namely, a, b, d, f, g and h, are reported from India, based on published literature and data from the Genbank. However, currently PCV2 vaccines are not available in India and are imported. This study reports the effect of different BEI concentrations on the inactivation of PCV2. The data generated indicated that complete inactivation of PCV2 infectivity was achieved at 3mM BEI within 8 hours at 37 °C and within 12 hours at 25 ºC. The temperature of 25 °C is preferred to avoid thermal degradation of the virus observed at 37 °C. The final concentration of 3mM of BEI during inactivation of PCV2 is within the accepted range used for inactivating viruses and in addition, the BEI is neutralized at the end of the inactivation process, minimizing risk of toxicity. The duration required for complete inactivation of viruses for vaccine preparation could be estimated based on Salk's determination of the minimum required inactivation time [11]. A safety margin to allow for intrinsic lower limit of detection (sensitivity) of the tissue culture system (PK15 cells) used for detection of residual replication of infective PCV2 virus should be considered. The "total inactivation time" [11] is defined as a total period equal to three times the incubation time with inactivating agent (here, BEI) required for total inactivation of virus (PCV2) infectivity as detected by the tissue culture system. The observation of this study, with a PK-15 cell culture based detection of infective PCV2, allows for estimation of total inactivation time required for 100 mL of $10^{6.3}$ $TCID_{50}$/mL PCV2 stock as 36 hours of incubation with 3mM BEI at 25 °C. This study could be used as a guideline for routine validation of the inactivation process of PCV2 with BEI, at varying volumes and batches of BEA used.

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