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EXPERT REVIEWS

# Inactivated virus vaccines from chemistry to prophylaxis: merits, risks and challenges

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The aim of this review is to make researchers aware of the benefits of an efficient quality control system for prediction of a developed vaccine's efficacy. Two major goals should be addressed when inactivating a virus for vaccine purposes: first, the infectious virus should be inactivated completely in order to be safe, and second, the viral epitopes important for the induction of protective immunity should be conserved after inactivation in order to have an antigen of high quality. Therefore, some problems associated with the virus inactivation process, such as virus aggregate formation, protein crosslinking, protein denaturation and degradation should be addressed before testing an inactivated vaccine *in vivo*.

**Keywords:** 2,2′-dithiodipyridine • β-propiolactone • binary ethylene imine • formaldehyde • gamma irradiation • glutaraldehyde • pH • temperature • UV • vaccine • virus inactivation

Classically, two main types of vaccines are used to protect man and animals clinically and virologically on challenge: modified live virus (attenuated virus) vaccines and killed virus (KV; inactivated virus) vaccines. KV vaccines are often preferred because of safety reasons, but some problems may be encountered using them. First, virus inactivation may turn out to be incomplete with outbreaks after vaccination as a consequence [1–3]. Second, the viral-neutralizing epitopes may be destroyed during inactivation, resulting in a poor neutralizing antibody response and a poor protection on challenge [4–6]. Therefore, efficient quality control of the inactivated antigen is necessary to evaluate virus inactivation and the effect of the inactivation procedure on neutralizing epitopes.

In the following paragraphs, different and most commonly used inactivation procedures for viruses will be discussed and their usefulness for the development of a KV vaccine will be evaluated. An overview of the discussed inactivation methods and the relevant chemical conditions involved is given in Table 1.

## Cross-linkers

### *Formaldehyde*

KV vaccine development based on inactivation with formaldehyde has been investigated for many viruses. A few of them are discussed below and presented in Table 2. Some formaldehyde-inactivated vaccines work well and protect against disease on challenge. This accounts for inactivated Ross River virus (RRV) [7], West Nile virus [8], dengue-2 virus [9], equine herpes virus type 1 [10], papilloma virus [11], Hantaan virus [12], influenza virus [13], hepatitis A virus [14], tick-borne encephalitis virus [15,16] and Japanese encephalitis virus [17]. However, with some vaccines inactivated in this way, problems have been encountered. In order to gain insight into the reasons for failure, a more detailed understanding of the chemistry is needed.

Formaldehyde (Figure 1A), also known as formalin when diluted in water, has an electron-deficient central carbon atom and is therefore electrophilic, as illustrated through one of the resonance forms. As a consequence, a nucleophile, such as a nonprotonated amino group, can attack the central carbonyl carbon. This chemical reaction is called a nucleophilic addition.

Formaldehyde has an effect on both genome and proteins. First of all, it monohydroxymethylates adenine [18]: the nonprotonated exocyclic amine on adenine (N6) acts as a nucleophile and reacts with the carbocation of formaldehyde [19]. Because of this nucleophilic addition, adenine is monohydroxymethylated, which blocks genome reading [20]. This

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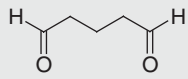
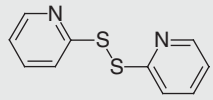
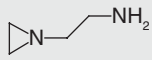
**Table 1. Overview of inactivation methods for the development of killed virus vaccines.**

| Method | Type | Mechanism | Ref. |
|---|---|---|---|
| Formaldehyde | Alkylating agent | Monohydroxymethylation of adenine | [18] |
| | | Crosslinking of RNA to capsid proteins, causing a block of genome reading | [25–27,107] |
| | Crosslinker | Crosslinking of proteins by formation of inter- and intra-molecular methylene bridges between hydroxymethylated amines | [20] |
| Glutaraldehyde  | Crosslinker | Crosslinking of proteins by the same mechanism as formaldehyde (described above) | [47] |
| 2,2´-dithiodipyridine  | Crosslinker | Crosslinking of proteins by oxidation of S-H groups causing formation of S-S bridges, which results in a covalent modification and functional inactivation of S-H-containing internal viral proteins | [54] |
| β-propiolactone | Alkylating agent | Alkylation of RNA and DNA as a consensus mechanism | [58] |
| | Crosslinker | Crosslinking of proteins | [59] |
| Binary ethylene imine  | Alkylating agent | Alkylation of RNA and DNA at low concentrations. Most likely genome reading is blocked by alkylation of guanine or adenine by binary ethylene imine | [72,73] |
| | | Alkylation of proteins (nucleocapsid) at high concentrations | [72] |
| pH | Denaturation agent | Denaturation of viral functionally active proteins | [80] |
| | RNA degradation | The close proximity of the hydroxyl group to the phosphor center of each internucleotide linkage facilitates transesterification under strongly acidic or strongly basic conditions, with a breakage of the phosphodiester bond as a consequence | [81] |
| Temperature | Denaturation agent | A high temperature denatures viral functionally active proteins | [82,83] |
| | RNA degradation | Virus inactivation at 'low' temperature (below 41°C) is considered to be caused by degradation of the nucleic acid | [84–86] |
| Gamma irradiation | Radiation | Viruses are inactivated primarily by direct damage, via disruption of the genome | [95] |
| | | Formation of free radicals that damage proteins | [95] |
| Ultraviolet light | Radiation | Induction of dimer formation between adjacent uracils in RNA. Dimer formation leads to pressure and breakage of the sugar backbone causing a block of genome reading | [89,90] |
| | | Works more slowly, ultraviolet light also causes structural modifications of the capsid proteins resulting in the formation of large and small photoproducts | [90,94] |

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monohydroxymethylation of adenine (Figure 1A), also known as alkylation, can occur on either DNA or RNA [21]. Monohydroxymethyladenine ($NH–CH_2OH$) is stable for days at room temperature and is more likely to exist than the Schiff base ($N=CH_2$) [22]. However, if adenine and formaldehyde are stored for days at room temperature and at a pH of 4.5 then methylene bis-adenine can be formed [19].

Formaldehyde can also react with nonprotonated amino groups of the N-terminal amino acid residue and the amino acids featuring N-containing functionality in their side chain, such as lysine, arginine, glutamine, tryptophan and histidine, or a sulfhydryl group in their side chain, such as cysteine. More specifically, as exemplary illustrated for the lysine side chain (Figure 1B), a nonprotonated amine acts as a nucleophile and reacts with the carbocation of formaldehyde. As a result, the amino group is monohydroxymethylated, as described above for adenine. The loss of a water molecule from the unstable hemiaminal results in Schiff base formation [23], also called an imine intermediate. The resulting Schiff base or imine intermediate can crosslink with arginine and tyrosine and to a lesser extent with glutamine, asparagine, tryptophan and histidine residues by a nucleophilic addition reaction [23]. In this way, inter- and intra-molecular methylene bridges can be formed (Figure 1B) [24]. Because of these bridges, proteins become inter- and intra-molecularly crosslinked.

In addition, these reactions can also cause crosslinking between genome and proteins through combining the above described

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**Table 2. Effect of formaldehyde on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs).**

| Study (year) | Genome | Envelope | Capsid | Virus family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| Pollock *et al.* (1982) | DNA (ss) | No | I | Parvo | CPV | Yes | Yes | NT | NT | NT | NT | [43] |
| | | | | Circo | No | | | | | | | |
| | DNA (ds) | Yes | I | Hepadna | No | | | | | | | |
| Skinner *et al.* (2000) | | | | Herpes | EHV-1 | Yes (0.04%) | NT | Yes | Yes | NT | Yes | [10] |
| | | | | Asfor | No | | | | | | | |
| | | | C | Pox | No | | | | | | | |
| | | No | I | Adeno | No | | | | | | | |
| Bell *et al.* (1994) | | | | Papova | Papilloma | Yes | NT | NT | NT | NT | Yes | [11] |
| | RNA (segmented, ss, -) | Yes | H | Arena | No | | | | | | | |
| Choi *et al.* (2003) | | | | Bunya | Hantaan | Yes (0.0185%) | NT | Yes | Yes | Yes | NT | [12] |
| Bahgat *et al.* (2009) | | | | Orthomyxo | Influenza | Yes | NT | Yes | NT | Yes | Yes | [13] |
| | RNA (nonsegmented, ss, -) | Yes | H | Filo | No | | | | | | | |
| Openshaw *et al.* (2001) | | | | Paramyxo | RSV | Yes | NT | Yes | Poor | NT | No | [34] |
| | | | | Rhabdo | No | | | | | | | |
| | | | | Borna | No | | | | | | | |
| Kistner *et al.* (2007) | RNA (nonsegmented, ss, +) | Yes | I | Toga | RRV | Yes (0.1%) | No | Yes | Yes | Yes | NT | [7] |
| Brown *et al.* (1993) | | | | | VEEV | Incomplete | NT | NT | NT | NT | No | [28] |
| Jahrling *et al.* (1984) | | | | | | Yes | NT | Yes | Yes | Yes | No | [6] |
| Delrue *et al.* (2009) | | | | Arteri | PRRSV | Yes ($1 \times 10^{-6}$%) | Yes | NT | NT | NT | NT | [41] |
| Ishizaka *et al.* (1999) | | | | Retro | SIV | Yes | NT | Yes | No | No | NT | [35] |
| Rossio *et al.* (1998) | | | | | HIV | Yes (1:80) | Yes | NT | NT | NT | NT | [42] |

+: Positive stranded; -: Negative stranded; C: Complex; CPV: Canine parvovirus; DEN: Dengue virus; ds: Double stranded; EHV: Equine herpes virus; FMDV: Foot-and-mouth disease virus; H: Helical; HAV: Hepatitis A virus; I: Icosahedral; JEV: Japanese encephalitis virus; NT: Not tested; PRRSV: Porcine reproductive and respiratory virus; PV: Poliovirus; RRV: Ross river virus; RSV: Respiratory syncytial virus; SARS-CoV: Severe acute respiratory syndrome coronavirus; ss: Single stranded; TBEV: Tick-borne encephalitis virus; VEEV: Venezuelan equine encephalitis virus; VN Ab: Virus neutralizing antibodies; VS Ab: Virus-specific antibodies; WNV: West Nile virus.

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**Table 2. Effect of formaldehyde on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs) (cont.).**

| Study (year) | Genome | Envelope | Capsid | Virus family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| Niedrig *et al.* (1993) | | | | | | Yes | NT | Yes | Yes | Yes | No | [5] |
| Putnak *et al.* (1996) | | | | Flavi | DEN-2 | Yes (0.05%) | NT | Yes | Yes | Yes | Yes | [9] |
| Appaiahgari *et al.* (2004) | | | | | JEV | Yes (1:40) | NT | Yes | Yes | NT | Yes | [17] |
| Samina *et al.* (2005) | | | | | WNV | Yes (0.1%) | NT | NT | NT | Yes | Yes | [8] |
| Goncharova *et al.* (2006) | | | | | TBEV | Yes | NT | Yes | Yes | NT | Yes | [15] |
| Qu *et al.* (2005) | | | H | Corona | SARS-CoV | Yes (1:2000) | NT | NT | Yes | NT | NT | [32] |
| Zhang *et al.* (2004) | | | | | | Yes (0.4%) | NT | Yes | Yes | NT | NT | [33] |
| Darnell *et al.* (2004) | | | | | | Incomplete | NT | NT | NT | NT | NT | [30] |
| | | No | I | Calici | No | | | | | | | |
| Patil *et al.* (2002) | | | | Picorna | FMDV | Yes (0.06%) | Yes | Poor | NT | NT | NT | [3] |
| King *et al.* (1981) | | | | | | Incomplete | NT | NT | NT | NT | No | [1] |
| Tano *et al.* (2007) | | | | | PV | Yes (0.025%) | Yes | NT | NT | NT | NT | [39] |
| Furesz *et al.* (1995) | | | | | HAV | Yes | NT | Yes | Yes | NT | Yes | [14] |
| | | | | Astro | No | | | | | | | |
| | RNA (segmented, ds) | No | I | Reo | No | | | | | | | |
| | | | | Birna | No | | | | | | | |

+: Positive stranded; -: Negative stranded; C: Complex; CPV: Canine parvovirus; DEN: Dengue virus; ds: Double stranded; EHV: Equine herpes virus; FMDV: Foot-and-mouth disease virus; H: Helical; HAV: Hepatitis A virus; I: Icosahedral; JEV: Japanese encephalitis virus; NT: Not tested; PRRSV: Porcine reproductive and respiratory virus; PV: Poliovirus; RRV: Ross river virus; RSV: Respiratory syncytial virus; SARS-CoV: Severe acute respiratory syndrome coronavirus; ss: Single stranded; TBEV: Tick-borne encephalitis virus; VEEV: Venezuelan equine encephalitis virus; VN Ab: Virus neutralizing antibodies; VS Ab: Virus-specific antibodies; WNV: West Nile virus.

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functionalities [25,26], which prevents the transcriptional machinery from reaching the genome [27].

One of the problems observed with formaldehyde is that some vaccines based on formaldehyde inactivation can contain incompletely inactivated virus particles, which can cause outbreaks of virus infections on vaccination [28]. This is reported for several viruses such as foot-and-mouth disease virus (FMDV) [1] and Venezuelan equine encephalitis virus (VEEV) [28]. Molecular analysis proved clearly that outbreaks of FMDV in western Europe in the 1980s and VEEV in central America in 1970s are a consequence of incompletely inactivated virus vaccines [28]. This can be due to crosslinking of nucleocapsid proteins with RNA. As a consequence, RNA cannot be degraded, and some virus particles escape from inactivation with formaldehyde [29]. It is also possible that the safety testing was not sufficient and that a higher concentration of formaldehyde is necessary to inactivate these viruses properly. Darnell *et al.* examined formaldehyde inactivation of the severe acute respiratory syndrome coronavirus (SARS-CoV) [30]. They showed that formaldehyde can inactivate the virus in a temperature-dependent manner. The virus could not be inactivated at a low temperature of 4°C even after 3 days. However, when using a higher temperature of 25 or 37°C, formaldehyde could inactivate most of the virus after 1 day [30]. Although not investigated in this particular study, in addition to temperature, the pH during inactivation can play a major role, as is further illustrated in this review (see 'pH' section). When SARS-CoV is treated with 0.05% formaldehyde for 48 h, it could be inactivated properly [31]. Vaccines based on formaldehyde-inactivated SARS-CoV are shown to induce virus-specific and virus-neutralizing antibodies [32,33]; however, some virus still remained infectious on day 3 of inactivation [30].

Some KV vaccines based on formaldehyde inactivation do not induce a virus-neutralizing antibody response and do not protect on challenge, for example respiratory syncytial virus (RSV) [34], inactivated HIV [5], SIV [35] and VEEV vaccines [6]. A poor immune response on vaccination can even lead to atypical or enhanced disease after infection of vaccinated recipients. This is most likely due to the poor induction of neutralizing antibody responses together with the formation of immune complexes between virus and antibodies, and further facilitated uptake and infection of these complexes by monocytic cells, termed antibody-dependent enhanced infection [36]. This is observed with inactivated measles [37] and RSV [34,38] vaccines.

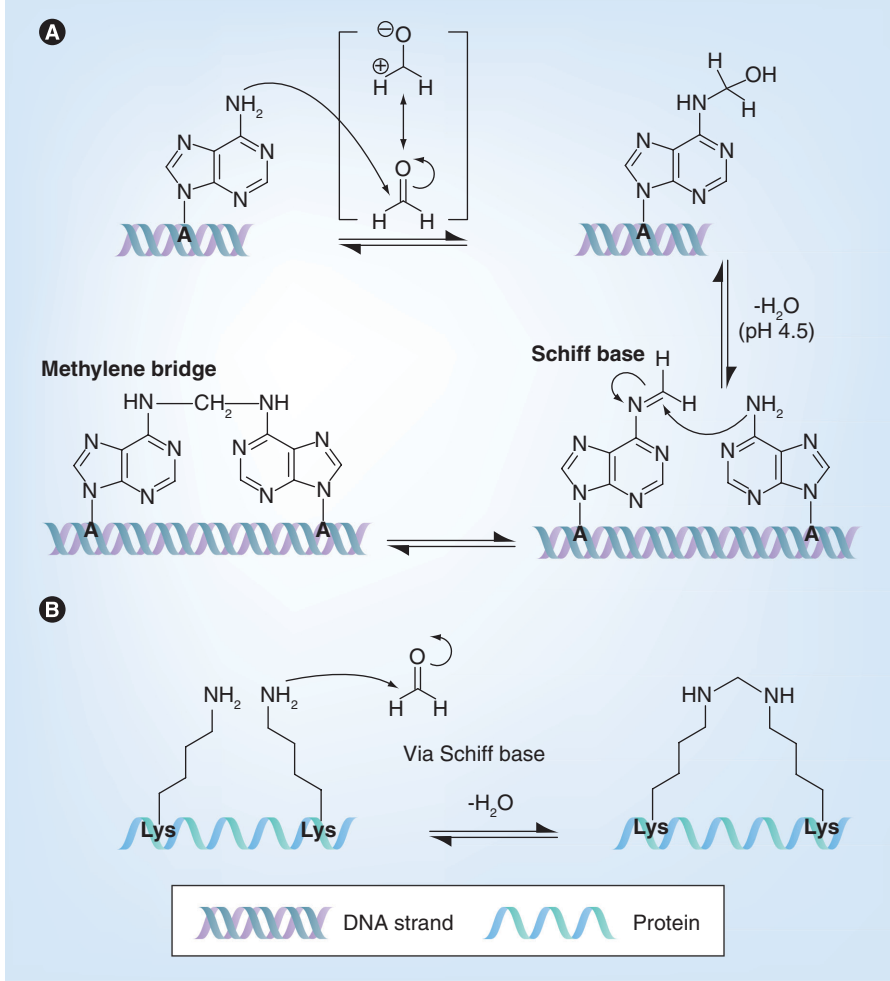


**Figure 1. Reaction mechanism of formaldehyde with either DNA/RNA or amino acids.** Reaction with **(A)** DNA or RNA (adenine) and, in a similar fashion, **(B)** amino acids (e.g., lysine) of proteins with a primary amine group: monohydroxy methylation and methylene bridge formation.
A: Adenine; Lys: Lysine.

Formaldehyde can destroy the viral structure, which can result in a poor immune response. When poliovirus (PV) is treated with formaldehyde, the extent of the modification of the antigenic sites is dependent on the strain used and correlates with the immune response that can be induced [39]. Treatment of porcine reproductive and respiratory syndrome virus (PRRSV) with formaldehyde inactivates the virus but does not preserve the viral entry-associated domains. These domains may be important for the induction of protective immunity, as neutralizing antibodies are directed to the viral epitopes that are involved in the attachment to the PRRSV receptors and internalization into the host cell [40]. Since crosslinking of viral entry-associated domains might interfere with the subsequent presentation of viral-neutralizing epitopes to cells of the adaptive immune system, this procedure is not useful for inactivated PRRSV vaccine development [41]. Modification of the virion structure on inactivation with formaldehyde is also reported for FMDV [3], HIV [42] and canine parvovirus [43].

In conclusion, although formaldehyde has been shown to correctly inactivate some viruses, given the problems discussed

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**Table 3. Effect of glutaraldehyde on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs).**

| Study (year) | Genome | Envelope | Capsid | Virus family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| | DNA (ss) | No | I | Parvo | No | | | | | | | |
| | | | | Circo | No | | | | | | | |
| Cham *et al.* (2006) | DNA (ds) | Yes | I | Hepadna | DHBV | Yes | NT | Yes | Yes | No | NT | [4] |
| | | | | Herpes | No | | | | | | | |
| | | | | Asfor | No | | | | | | | |
| | | | C | Pox | No | | | | | | | |
| | | No | I | Adeno | No | | | | | | | |
| | | | | Papova | No | | | | | | | |
| | RNA (segmented, ss, -) | Yes | H | Arena | No | | | | | | | |
| | | | | Bunya | No | | | | | | | |
| | | | | Orthomyxo | No | | | | | | | |
| | RNA (nonsegmented, ss, -) | Yes | H | Filo | No | | | | | | | |
| Keles *et al.* (1998) | | | | Paramyxo | BRSV | Yes (0.075%) | NT | Yes | Yes | Yes | NT | [51] |
| | | | | Rhabdo | No | | | | | | | |
| | | | | Borna | No | | | | | | | |
| | RNA (nonsegmented, ss, +) | Yes | I | Toga | No | | | | | | | |
| | | | | | No | | | | | | | |
| Delrue *et al.* (2009) | | | | Arteri | PRRSV | Yes (1 × $10^{-6}$%) | Yes | NT | NT | NT | NT | [41] |
| Darnell *et al.* (2004) | | Yes | H | Corona | SARS-CoV | Yes (1:4000) | NT | NT | NT | NT | NT | [30] |
| | | | | Retro | No | | | | | | | |
| | | | | Flavi | No | | | | | | | |

+: Positive stranded; -: Negative stranded; BRSV: Bovine respiratory syncytial virus; C: Complex; DHBV: Duck HBV; ds: Double stranded; H: Helical; I: Icosahedral; IBDV: Infectious bursal disease virus; NT: Not tested; PRRSV: Porcine reproductive and respiratory syndrome virus; SARS-CoV: Severe acute respiratory syndrome coronavirus; ss: Single stranded; VN Ab: Virus-neutralizing antibodies; VS Ab: Virus-specific antibodies.

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**Table 3. Effect of glutaraldehyde on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs) (cont.).**

| Study (year) | Genome | Envelope | Capsid | Virus family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| | | No | I | Calici | No | | | | | | | |
| | | | | Picorna | No | | | | | | | |
| | | | | Astro | No | | | | | | | |
| | RNA (segmented, ds) | No | I | Reo | No | | | | | | | |
| Cepica *et al.* (1998) | | | | Birna | IBDV | Yes (0.01%) | Yes | NT | NT | NT | NT | [52] |

+: Positive stranded; -: Negative stranded; BRSV: Bovine respiratory syncytial virus; C: Complex; DHBV: Duck HBV; ds: Double stranded; H: Helical; I: Icosahedral; IBDV: Infectious bursal disease virus; NT: Not tested; PRRSV: Porcine reproductive and respiratory syndrome virus; SARS-CoV: Severe acute respiratory syndrome coronavirus; ss: Single stranded; VN Ab: Virus-neutralizing antibodies; VS Ab: Virus-specific antibodies.

above, care must be taken regarding the exact protocol used. In part, some failures could be due to an insufficient inactivation protocol. Problems, such as incomplete virus inactivation, that lead to outbreaks have to be kept in mind when using this inactivation method for KV vaccine development. Formaldehyde can be used for KV development for some viruses, but several KV vaccines based on formaldehyde inactivation do not protect the host on challenge with virulent virus. This could be due to RNA–protein crosslinking [26,27] or modification of viral proteins by formaldehyde, as reported for PV, FMDV and HIV [3,39,42].

### *Glutaraldehyde*

Examples of virus inactivation with glutaraldehyde are summarized in Table 3. Although there are few examples, complete virus inactivation with glutaraldehyde seems to be less problematic than with formaldehyde.

Glutaraldehyde (Figure 2) is a saturated, five-carbon dialdehyde. The carbonyl carbons are electrophilic and therefore a nucleophile, such as a nonprotonated amino group, might attack these carbons in a nucleophilic addition reaction, just like formaldehyde.

Genomic DNA or RNA is the target of glutaraldehyde. Although the exact mechanism of action has not clearly been described, it is likely that glutaraldehyde acts in the same way as formaldehyde, since similar chemical groups are present on formaldehyde and glutaraldehyde [44]. The exocyclic amino group on adenine (N6) is the reactive group, which attacks glutaraldehyde [45]. It is certain that glutaraldehyde can have an effect on the genome, since RNA and protein synthesis can be blocked by this aldehyde [46].

Glutaraldehyde can have an effect on proteins as well, because nonprotonated amines of amino acids, such as lysine, can be crosslinked with each other [47]. However, the reaction mechanism of glutaraldehyde with proteins is different from that of formaldehyde (Figure 2). First, glutaraldehyde forms unsaturated aldehydes by aldol condensation and elimination of water [48]. Then, several amino acids can be linked to glutaraldehyde via a Michael-type addition, the nonprotonated amines acting as nucleophiles and reacting with the double bond of the glutaraldehyde oligomer [49]. In this way, the amino acids are coupled by formation of a bridge [50]. Unlike the reaction with formaldehyde, intermediate Schiff bases or imines are not formed [44,50], but both pathways result in covalently linked amino acid side chains.

In addition, the reactions described above can also cause crosslinking of the genome and proteins [25], which blocks genome reading [27].

To investigate virus inactivation with glutaraldehyde, SARS-CoV was incubated with glutaraldehyde at two different dilutions and at different temperatures. The glutaraldehyde exhibited a temperature dependence in its ability to inactivate SARS-CoV. The virus could not be inactivated by glutaraldehyde at 4°C even after 3 days. However, when a higher temperature of 25 or 37°C was used, the virus could be inactivated in 2 and 1 days, respectively [30].

As glutaraldehyde is a bi-functional or, after aldol condensation, multifunctional crosslinking agent, virus damage might be more

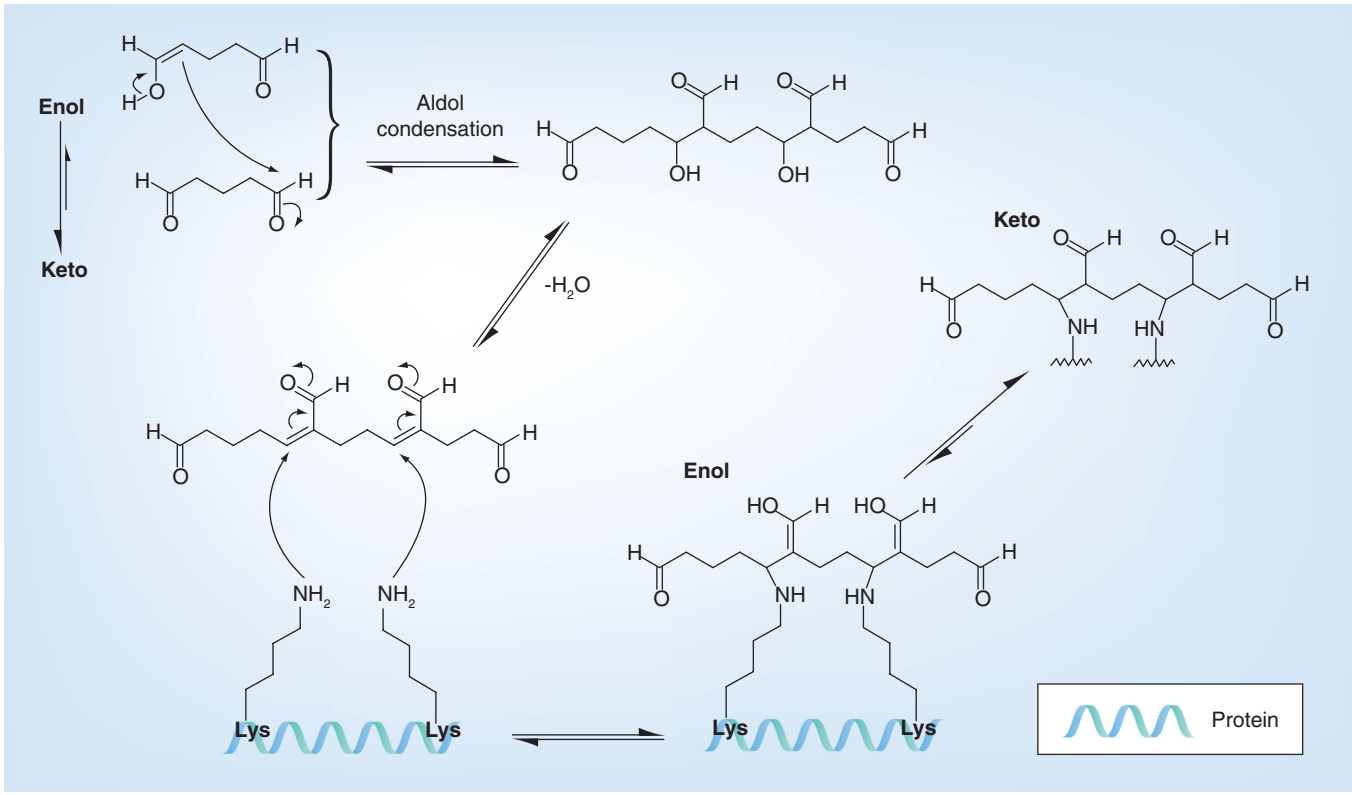


**Figure 2. Reaction mechanism of glutaraldehyde with amino acids of proteins (e.g., with the primary amine group of lysine).**
Lys: Lysine.

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extensive. Therefore, the induction of a protective immune response can be more of a problem with glutaraldehyde-inactivated virus vaccines. Duck HBV (DHBV) and bovine RSV (BRSV) are viruses for which the use of glutaraldehyde has been tested for the production of a KV vaccine [4,51]. Glutaraldehyde-inactivated DHBV and BRSV developed virus-specific antibodies, but ducks vaccinated with glutaraldehyde-inactivated DHBV and lambs vaccinated with glutaraldehyde-inactivated BRSV were not fully protected against viremia following challenge. This indicates that there was no induction of protective immunity in these animals [4,51].

The destruction of the viral structure by glutaraldehyde may be a problem. Similar to formaldehyde, glutaraldehyde could inactivate PRRSV but did not preserve the viral entry-associated domains and is therefore not useful for inactivated PRRSV vaccine development [41]. Glutaraldehyde was also shown to change the conformational structure of infectious bussal disease virus [52].

In conclusion, glutaraldehyde can inactivate viruses properly, but there is not always protection against infection on challenge for the KV vaccines discussed here. This could be due to destruction of viral proteins by glutaraldehyde.

### *Aldrithiol or 2,2´-dithiodipyridine*

Examples of virus inactivation with aldrithiol, dithiodipyridine (AT-2), are summarized in Table 4. This procedure for virus inactivation for KV vaccines has been investigated for HIV [42], PRRSV [41] and RSV [53].

AT-2 oxidizes S-H groups, which results in formation of S-S bridges that crosslink proteins (Figure 3). As the aromatic thiolate is the better leaving group, the reaction equilibrium is shifted toward the aliphatic disulfides. The internal viral proteins are subjected to the intracellular (reducing) environment, and as a consequence the cysteine residues are present in thiol form (S-H). By contrast, the surface proteins of viruses are subjected to the extracellular (oxidizing) environment, and as a consequence, the cysteines are present as disulfides (S-S). Treatment with AT-2 results in covalent modification and functional inactivation of S-H-containing internal viral proteins, such as the nucleocapsid protein, which is required for HIV infectivity, whereas the envelope glycoproteins with disulfide bonded cysteines remain unaffected [54].

HIV preparations inactivated with AT-2 did not retain detectable infectivity, but showed maintenance of conformational and functional integrity. This suggests that such virions may be useful as vaccine antigens [42]. An RSV vaccine based on AT-2 inactivation was found to be effective in the protection of cotton rats against virus replication in the lungs. This suggests that compounds that inactivate retroviruses by targeting the zinc finger motif in their nucleocapsid proteins are also effective against RSV [53].

However, AT-2 cannot inactivate all viruses. PRRSV is not sensitive for AT-2 and could not be inactivated by it [41]. For PRRSV, disulfide brigdes between nucleocapsid proteins are important for virus infection, but as stated above, these disulfide bridges remain unaffected by AT-2 [55]. It seems there are no free thiol groups in

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**Table 4. Effect of 2,2′-dithiodipyridine on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs).**

| Study (year) | Genome | Envelope | Capsid | Virus family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| | DNA (ss) | No | I | Parvo | No | | | | | | | |
| | | | | Circo | No | | | | | | | |
| | DNA (ds) | Yes | I | Hepadna | No | | | | | | | |
| | | | | Herpes | No | | | | | | | |
| | | | | Asfor | No | | | | | | | |
| | | | C | Pox | No | | | | | | | |
| | | No | I | Adeno | No | | | | | | | |
| | | | | Papova | No | | | | | | | |
| | RNA (segmented, ss, -) | Yes | H | Arena | No | | | | | | | |
| | | | | Bunya | No | | | | | | | |
| | | | | Orthomyxo | No | | | | | | | |
| | RNA (nonsegmented, ss, -) | Yes | H | Filo | No | | | | | | | |
| Boukhvalova *et al.* (2009) | | | | Paramyxo | RSV | Yes (10 mM) | No | NT | NT | Yes | NT | [53] |
| | | | | Rhabdo | No | | | | | | | |
| | | | | Borna | No | | | | | | | |
| | RNA (nonsegmented, ss, +) | Yes | I | Toga | No | | | | | | | |
| | | | | | No | | | | | | | |
| Delrue *et al.* (2009) | | | | Arteri | PRRSV | No (2 mM) | No | NT | NT | NT | NT | [41] |
| Rossio *et al.* (1998) | | | | Retro | HIV | Yes (300 µM) | No | NT | NT | NT | NT | [42] |
| Lifson *et al.* (2004) | | | | | SIV | Yes (1 mM) | NT | Yes | Yes | Yes | NT | [56] |

+: Positive stranded; -: Negative stranded; C: Complex; ds: Double stranded; H: Helical; I: Icosahedral; NT: Not tested; PRRSV: Porcine reproductive and respiratory virus; RSV: Respiratory syncytial virus; SIV: Simian immunodeficiency virus; ss: Single stranded; VN Ab: Virus-neutralizing antibodies; VS Ab: Virus-specific antibodies.

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**Table 4. Effect of 2,2′-dithiodipyridine on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs) (cont.).**

| Study (year) | Genome | Envelope | Capsid | Virus family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| Vanhee *et al.* (2009) | | | | | | Yes (300 μM) | NT | No | No | Yes | NT | [57] |
| | | | | Flavi | No | | | | | | | |
| | | | H | Corona | No | | | | | | | |
| | | No | I | Calici | No | | | | | | | |
| | | | | Picorna | No | | | | | | | |
| | | | | | No | | | | | | | |
| | | | | Astro | No | | | | | | | |
| | RNA (segmented, ds) | No | I | Reo | No | | | | | | | |
| | | | | Birna | No | | | | | | | |

+: Positive stranded; -: Negative stranded; C: Complex; ds: Double stranded; H: Helical; I: Icosahedral; NT: Not tested; PRRSV: Porcine reproductive and respiratory virus; RSV: Respiratory syncytial virus; SIV: Simian immunodeficiency virus; ss: Single stranded; VN Ab: Virus-neutralizing antibodies; VS Ab: Virus-specific antibodies.

PRRSV that are important for infection. Probably, only viruses where free thiol groups are important for virus infection can be inactivated by AT-2.

There are also some vaccination studies where no full protection after vaccination can be assured. A vaccination study with AT-2 inactivated SIV showed that an immune response could be induced, and there was virological protection on challenge with homologous, but not heterologous, virus [56]. Another study could not detect an immune response before challenge. There was an antibody response on challenge, but vaccination only partly protected against viremia and infection on challenge [57].

In conclusion, AT-2 seems to inactivate HIV and SIV properly with preservation of the virion structure, but vaccination studies cannot prove that AT-2 inactivated virus can protect the host on challenge. AT-2 was not effective with other viruses.

## Alkylating agents

### *β-propiolactone*

β-propiolactone (BPL) is widely used to inactivate viruses for vaccine purposes (Table 5), mainly acting as an alkylating agent on guanine of viral DNA or RNA [58,59]. As illustrated in Figure 4, the nucleophilic guanine of nucleic acids reacts with the electrophilic BPL through a nucleophilic substitution mechanism causing the ring opening of the strained BPL and the *N*-alkylation of guanine. As BPL is much more toxic as a carcinogen [60] and displays the same mode of action as binary ethylene imine (BEI) discussed in the section 'Aminoethyl ethylene imine' the interested reader is referred to the more specialized literature. Interestingly, the mechanistic details and the chemical modifications of BPL treatment have recently been elucidated through systematic studies on nucleobase, nucleoside and peptide model systems, revising the above G-alkylation consensus pathway and providing a more nuanced view on the chemistry involved [59]. In fact, acylation and cross-linking reactions also occur, to a significant extent, with proteins (cysteine, methionine and histidine as most reactive residues) being modified more extensively than originally agreed from earlier literature, which can have serious implications for vaccine production. Indeed, modifications on amino acids by BPL treatment can, for example, lead to the disability of influenza viruses to enter cells by virus–membrane fusion and modification of hemagglutinin [61,62].

BPL-inactivated virus vaccines against yellow fever [63], SARS-CoV [64], influenza [65] and rabies [66] were able to induce the production of neutralizing antibodies. However, BPL-inactivated influenza showed reduced hemagglutinin and neuraminidase activity [62]. Vaccines based on BPL-inactivated virus against SARS-CoV [64], hematopoietic necrosis virus (HNV) [67] and influenza [65], were able to protect on challenge.

In conclusion, BPL seems to inactivate viruses properly and is able to induce a protective immune response. This can be expected, as BPL can alkylate the viral genome. However, protein modification should be kept in mind when using BPL.

### *Aminoethyl ethylene imine*

Virus inactivation by ethylene imines was first introduced over 30 years ago [68,69]. Originally, two chemically similar compounds,



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ethylene imine (aziridine) and acetylethylene imine, were proposed for use in the development of KV vaccines [70,71]. However, the selectivity of aziridine toward nucleic acids is not very high. To improve this selectivity, a product with more protonizable amino groups was necessary, and aminoethyl ethylene imine, also called BEI, which has two protonizable amino groups while aziridine only has one, was selected.

If a low concentration of BEI is used, the virus capsid is not alkylated, but BEI passes through the capsid and alkylates the genome [72]. N7-guanine of the genome acts as a nucleophile and reacts with the electrophile BEI. The nucleophilic substitution reaction performed by N7-guanine causes an opening of the BEI ring and guanine becomes alkylated (Figure 5) [73]. In addition, adenine is alkylated via a ring-opening reaction with BEI [72]. This ring-opening reaction in RNA nucleosides is approximately two- to three-times faster than that in DNA nucleosides [74]. So far, there are no reports on any interaction of BEI with proteins, at least if low concentrations are used, which suggests that the neutralizing viral epitopes are preserved after treatment of a virus with BEI.

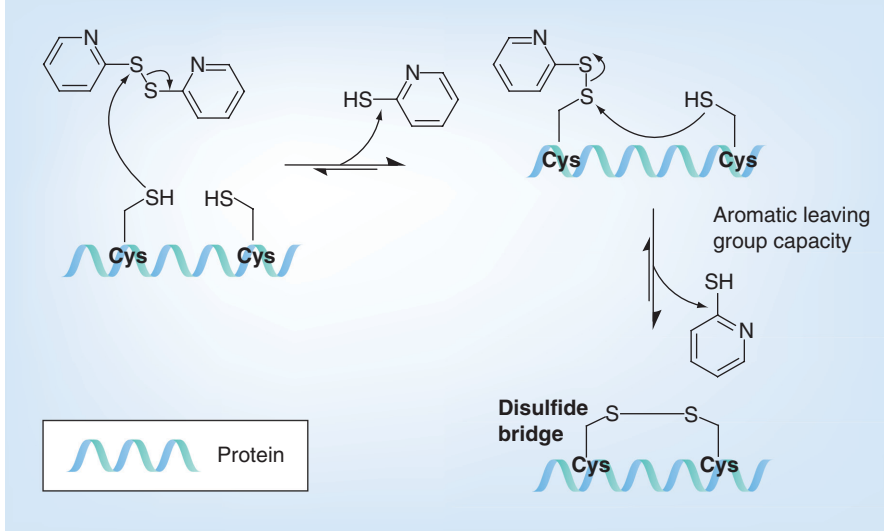


**Figure 3. Reaction mechanism of 2,2′-dithiodipyridine with cysteine.**
Cys: Cysteine.

BEI is used for the inactivation of many viruses, such as FMDV [70], RRV [75], sheep pox [76], HIV [77], Nipah virus [78], HNV [67] and PRRSV (Table 6) [79].

As mentioned above, the preservation of the viral structure should not be a problem if a low concentration of BEI is used. This has also been shown for PRRSV, where BEI could inactivate PRRSV with the preservation of the viral entry-associated domains [41]. Furthermore, the inactivation of FMDV with BEI did not affect the antigenity of FMDV [70].

Most vaccines based on BEI-inactivated virus are able to induce a protective immune response. Mice vaccinated with a purified BEI-inactivated RRV vaccine were protected against challenge with live virus. The vaccine induced significant levels of neutralizing antibody in all strains of mice tested [75]. A local Egyptian sheep pox virus strain inactivated with BEI could induce virus-specific antibodies starting from 1-week post-vaccination until 4-weeks post-challenge. Vaccinated lambs were protected on challenge up to 6-months post-vaccination [76]. Race *et al.* have shown that the experimental BEI-inactivated HIV vaccine induces virus-neutralizing antibodies against both the homologous vaccine strain and a heterologous virus strain [77]. Also, virus-specific antibodies could be induced against BEI-inactivated Nipah virus; however, these did not neutralizing the virus *in vitro* [78]. After vaccination of pigs with BEI-inactivated PRRSV, they induced a virus-specific and virus-neutralize antibody response, which was able to reduce viremia after challenge [79]. However, rainbow trout vaccinated with BEI-inactivated HNV and challenged with live HNV showed no statistically significant differences between the cumulative percentage mortality in the group of fish immunized with the BEI vaccines and the mortality of fish in the mock-vaccinated control groups when tested 28 or 56 days after vaccination [67].

In conclusion, BEI seems to inactivate viruses properly and most KV vaccines based on BEI inactivation protect the host after challenge with virulent virus, which is expected since BEI does not interfere with the virus structure if a low concentration is used. Only one KV vaccine did not protect the host on challenge, but the possible reason for this remains to be elucidated.

## Denaturing procedures

### *pH*

Increasing or decreasing the pH has an effect on proteins by protein denaturation. This means that the proteins adopt a different 3D structure. A low (acidic) or a high (alkaline) pH can inactivate viruses via denaturation of the secondary structures of proteins, thereby altering the conformation of viral proteins that are involved in attachment to and replication in a host cell. The conformation

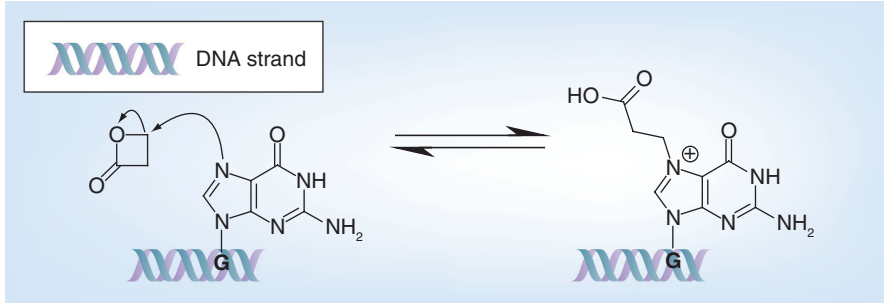


**Figure 4. Reaction mechanism of β-propiolactone with DNA or RNA (guanine).**
G: Guanine.

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**Table 5. Effect of β-propiolactone on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs).**

| Study (year) | Genome | Envelope | Capsid | Virus family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| | DNA (ss) | No | I | Parvo | No | | | | | | | |
| | | | | Circo | No | | | | | | | |
| | DNA (ds) | Yes | I | Hepadna | No | | | | | | | |
| | | | | Herpes | No | | | | | | | |
| | | | | Asfor | No | | | | | | | |
| | | | C | Pox | No | | | | | | | |
| | | No | I | Adeno | No | | | | | | | |
| | | | | Papova | No | | | | | | | |
| | RNA (segmented, ss, -) | Yes | H | Arena | No | | | | | | | |
| | | | | Bunya | No | | | | | | | |
| Bodewes *et al.* (2010) | | | | Orthomyxo | Influenza | Yes | NT | Yes | Yes | Yes | Yes | [65] |
| Jonges *et al.* (2010) | | | | | | Yes | Yes | NT | NT | NT | NT | [62] |
| | RNA (nonsegmented, ss, -) | Yes | H | Filo | No | | | | | | | |
| | | | | Paramyxo | No | | | | | | | |
| Anderson *et al.* (2008) | | | | Rhabdo | HNV | Yes (2.7 mM) | NT | NT | NT | NT | Yes | [67] |
| Sabchareon *et al.* (1999) | | | | | Rabies | Yes | NT | Yes | Yes | NT | NT | [66] |
| | | | | Borna | No | | | | | | | |
| | RNA (nonsegmented, ss, +) | Yes | I | Toga | No | | | | | | | |
| | | | | | No | | | | | | | |
| | | | | Arteri | No | | | | | | | |
| | | | | Retro | No | | | | | | | |

+: Positive stranded; -: Negative stranded; C: Complex; SARS-CoV: Severe acute respiratory syndrome coronavirus; ds: Double stranded; H: Helical; HNV: Hematopoietic necrosis virus; I: Icosahedral; NT: Not tested; RRV: Ross river virus; ss: Single stranded; VN Ab: Virus-neutralizing antibodies; VS Ab: Virus-specific antibodies.

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**Table 5. Effect of β-propiolactone on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs) (cont.).**

| Study (year) | Genome | Envelope | Capsid | Virus family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| Monath *et al.* (2011) | | | | Flavi | Yellow fever | Yes | NT | Yes | Yes | NT | NT | [63] |
| Roberts *et al.* (2010) | | | H | Corona | SARS-CoV | Yes | NT | Yes | Yes | Yes | NT | [64] |
| | | No | I | Calici | No | | | | | | | |
| | | | | Picorna | No | | | | | | | |
| | | | | | No | | | | | | | |
| | | | | Astro | No | | | | | | | |
| | RNA (segmented, ds) | No | I | Reo | No | | | | | | | |
| | | | | Birna | No | | | | | | | |

+: Positive stranded; -: Negative stranded; C: Complex; SARS-CoV: Severe acute respiratory syndrome coronavirus; ds: Double stranded; H: Helical; HNV: Hematopoietic necrosis virus; I: Icosahedral; NT: Not tested; RRV: Ross river virus; ss: Single stranded; VN Ab: Virus-neutralizing antibodies; VS Ab: Virus-specific antibodies.

of spike proteins of coronaviruses for instance changes at pH 8 and, as a result, entry of the virus into the host cell is initiated, but a lower or higher pH is associated with a loss of reactivity [80].

The pH also has an effect on the genomic level (Figure 6). The close proximity of the hydroxyl group of RNA to the phosphorous center of each internucleotide linkage facilitates transesterification under strongly acidic or strongly basic conditions [81]. Base-catalyzed reactions proceed via a nucleophilic addition mechanism where the oxygen attacks the adjacent phosphorus center, with a breakage of the phosphodiester bond as a consequence (Figure 6).

To date, there are no reports about the use of extreme pH conditions for the inactivation of viruses for KV vaccines. However, inactivation kinetics were performed for SARS-CoV [30]. The infectivity of SARS-CoV is sensitive to pH extremes, as moderate pH variations from 5 to 9 could not inactivate the virus, whereas extremely alkaline (pH 12 and 14) or acidic (pH 1 and 3) conditions could inactivate the virus (Table 7).

Preserving the viral structure is a problem if viruses are inactivated under extreme pH conditions. PRRSV inactivation by changing the pH can inactivate the virus, but does not preserve the viral entry-associated domains on PRRSV (Table 7). This suggests that, at least in the case of PRRSV, inactivation by changing the pH is not suitable to generate a proper KV vaccine [41].

In conclusion, extreme pH can inactivate viruses, but the effect of a KV vaccine based on this inactivation method in terms of protection against challenge is unknown. It is possible that the outcome of a vaccine based on pH inactivation will give a poor immune response, as the virus structure can be destroyed.

### *Temperature*

By heating or pasteurizing viruses, they can become inactivated by denaturation of the secondary and/or tertiary structures of the viral proteins. It is possible that the conformation of the viral proteins involved in attachment to and replication in a host cell is changed in this process [82,83]. Thermal inactivation of viruses by RNA degradation via breakage of the phosphodiester bond has also been described (Figure 6) [84]. Virus inactivation at 'low' temperatures (below 41°C) is considered to be caused by degradation of the nucleic acid, whereas virus inactivation at 'high' temperature is related to protein denaturation [84–86].

Heat inactivation has been studied as a method to develop a KV vaccine for SARS-CoV [30], HIV [87] and HNV (Table 7) [67]. To test the ability of heat to inactivate the SARS-CoV, Darnell *et al.* incubated the virus at three temperatures (56, 65 and 75°C) for increasing periods of time [30]. They found that at 56 and 65°C most of the virus was inactivated. However, some virus remained infectious at a level close to the detection limit for the assay, suggesting that some virus particles were stable at 56 and 65°C. One possible explanation for this result may be the presence of aggregates that slowly dissociate. Although the virus was incompletely inactivated at 56 and 65°C, even at 60 min of incubation, it was completely inactivated at 75°C after 45 min of incubation [30]. Taken together, these results suggest that viral inactivation by pasteurization might be very effective if the treatment is long enough.



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**Table 6. Effect of binary ethylene imine on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs).**

| Study (year) | Genome | Envelope | Capsid | Virus family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| | DNA (ss) | No | I | Parvo | No | | | | | | | |
| | | | | Circo | No | | | | | | | |
| | DNA (ds) | Yes | I | Hepadna | No | | | | | | | |
| | | | | Herpes | No | | | | | | | |
| | | | | Asfor | No | | | | | | | |
| Awad *et al.* (2003) | | | C | Pox | Sheep pox | Yes (2%) | NT | Yes | Yes | NT | Yes | [76] |
| | | No | I | Adeno | No | | | | | | | |
| | | | | Papova | No | | | | | | | |
| | RNA (segmented, ss, -) | Yes | H | Arena | No | | | | | | | |
| | | | | Bunya | No | | | | | | | |
| | | | | Orthomyxo | No | | | | | | | |
| | RNA (nonsegmented, ss, -) | Yes | H | Filo | No | | | | | | | |
| Berhane *et al.* (2006) | | | | Paramyxo | Nipah virus | Yes (3 mM) | NT | Yes | No | NT | NT | [78] |
| Anderson *et al.* (2008) | | | | Rhabdo | HNV | Yes (1.5 mM) | NT | NT | NT | NT | No | [67] |
| | | | | Borna | No | | | | | | | |
| Aaskov *et al.* (1997) | RNA (nonsegmented, ss, +) | Yes | I | Toga | RRV | Yes | NT | Yes | Yes | Yes | NT | [75] |
| Delrue *et al.* (2009); Vanhee *et al.* (2009) | | | | Arteri | PRRSV | Yes (1 mM) | No | Yes | Yes | Yes | Yes | [41,79] |

+: Positive stranded; -: Negative stranded; C: Complex; ds: Double stranded; FMDV: Foot-and-mouth disease virus; H: Helical; HNV: Hematopoietic necrosis virus; I: Icosahedral; NT: Not tested; PRRSV: Porcine reproductive and respiratory syndrome virus; RRV: Ross river virus; ss: Single stranded; VN Ab: Virus-neutralizing antibodies; VS Ab: Virus-specific antibodies.

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**Table 6. Effect of binary ethylene imine on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs) (cont.).**

| Study (year) | Genome | Envelope | Capsid | Virus family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| Race *et al.* (1995) | | | | Retro | HIV | Yes (10 mM) | No | Yes | Yes | NT | NT | [77] |
| | | | | Flavi | No | | | | | | | |
| | | | H | Corona | No | | | | | | | |
| | | No | I | Calici | No | | | | | | | |
| Bahnemann *et al.* (1975) | | | | Picorna | FMDV | Yes (1.5 mM) | No | NT | NT | NT | NT | [70] |
| | | | | | No | | | | | | | |
| | | | | Astro | No | | | | | | | |
| | RNA (segmented, ds) | No | I | Reo | No | | | | | | | |
| | | | | Birna | No | | | | | | | |

+: Positive stranded; -: Negative stranded; C: Complex; ds: Double stranded; FMDV: Foot-and-mouth disease virus; H: Helical; HNV: Hematopoietic necrosis virus; I: Icosahedral; NT: Not tested; PRRSV: Porcine reproductive and respiratory syndrome virus; RRV: Ross river virus; ss: Single stranded; VN Ab: Virus-neutralizing antibodies; VS Ab: Virus-specific antibodies.

For the preservation of the viral structure, a high temperature is suggested to have an effect on the viral proteins, whereas an inactivation temperature below 41°C is suggested to have no effect on protein level [84–86]. *In vitro* infection assays and western blot analysis showed that in the case of PRRSV the entry-associated domains were indeed preserved after treatment at 37°C and that the virus did not replicate anymore [41]. However, heat-inactivated influenza virus (>55°C) lost hemagglutinin and neuraminidase activity [62].

When it comes to induction of neutralizing antibodies and protection after vaccination, the outcome is vaccine dependent. When BALB/c mice and rhesus macaques (*Macaca mulatta*) were vaccinated with HIV samples inactivated at 62°C, virus-specific antibodies were observed after the first vaccination. In addition, the sera contained antibodies capable of neutralizing the vaccine strain [87]. In another study, HNV inactivated at 50°C did not protect fish against challenge. The percentage mortality of fish in the vaccinated groups was not statistically different compared with the mortality of fish in either the mock-vaccinated or unhandled control groups [67].

In conclusion, it has been shown that temperature can inactivate viruses, but some heat-resistant particles can remain in the inactivated samples. Some heat-inactivated vaccines could induce a neutralizing antibody response, whereas others could not prevent disease. This is probably due to protein modification, because a high inactivation temperature was used. The inactivation temperature should be below 41°C if protein modifications are not desirable.

## Irradiation

### *Ultraviolet light*

Based on wavelength, Ultraviolet (UV) light is subdivided into three classifications: UVA (320–400 nm), UVB (280–320 nm) and UVC (200–280 nm) [30]. UVC is absorbed by RNA and DNA bases and can lead to formation of dimers between two adjacent pyrimidines (uracil and thymine) (Figure 7). UVB can also induce formation of pyrimidine dimers, but is 20- to 100-fold less efficiently than UVC [88]. UVA is weakly absorbed by RNA and DNA and forms pyrimidine dimers much less efficiently than UVB and UVC [88].

Thus, in general, UV causes formation of pyrimidine dimers between two adjacent or opposing pyrimidines [89,90]. The two pyrimidines react through a pericyclic $2\pi$–$2\pi$ cycloaddition reaction resulting in the formation of a cyclobutane ring (Figure 7). The dimers cause a certain strain in the sugar backbone of the genome, which possibly leads to breaks in the genome. In addition, the uracil dimers formed by UV irradiation inactivate the RNA molecule as a transcription template [91].

This procedure for virus inactivation has been tested to develop a KV vaccine against viruses such as rabbit hemorrhagic disease virus (RHDV) [92], murine leukemia virus strain Cas-Br-M [93] and PRRSV [79] and are summarized in Table 8.

Some vaccines based on UV-inactivated viruses are able to induce an immune response. UV-inactivated murine leukemia virus strain Cas-Br-M (UV-Cas), induced a strong, Cas-specific cytotoxic

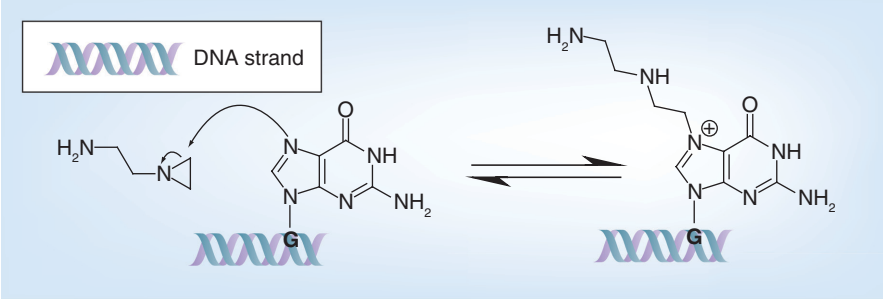


**Figure 5. Reaction mechanism of binary ethylene imine with DNA or RNA (guanine).**
G: Guanine.

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T-lymphocyte response in newborn NFS/N mice. Vaccination with UV-Cas protected mice against disease and reduced virus replication [93]. In addition, vaccination of pigs with UV-inactivated PRRSV induced virus-specific and virus-neutralizing antibody responses, which were able to reduce viremia after challenge [79].

However, there are also UV-inactivated vaccines that do not give protection against virus infection. Rabbits that were vaccinated with UV-inactivated RHDV and challenged with virulent virus, all died within 82 h after challenge. No antibodies were detected at the time of death. These findings show that vaccination with UV-inactivated RHDV does not protect rabbits against challenge with virulent virus in this study [92].

In terms of the preservation of the viral structure after UV inactivation, caution is necessary. UV could inactivate PRRSV with preservation of the viral entry-associated domains [41], but this is not the case for all viruses. The infectivity of mengovirus is lost very rapidly on exposure to UV irradiation, probably due to dimer formation in the viral RNA [90]. Besides this rapid effect on RNA and DNA, UV irradiation also causes structural modifications of the capsid proteins, resulting in the formation of large and small photoproducts, which is a much slower process [90]. The formation of large photoproduct proteins has also been reported for UV-irradiated reovirus [94].

In conclusion, it has been shown that UV irradiation inactivates viruses properly. If the virus structure is preserved after inactivation, the KV vaccine based on this UV-inactivated virus can protect the host on challenge. Some KV vaccines based on UV inactivation, however, do not protect the host after challenge with virulent virus. This might be due to the formation of photoproducts that interfere with the viral proteins.

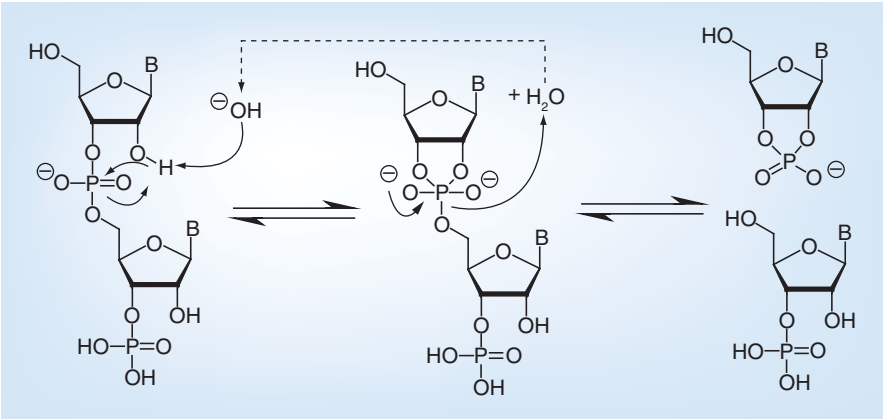


**Figure 6. RNA degradation in an alkaline environment.**
B: Nucleobase.

### *Gamma irradiation*

There are two mechanisms by which gamma irradiation can inactivate biological material. The first mechanism is a direct result of a photon depositing energy into the target. The transfer of this energy results in the dislocation of electrons and breakage of covalent bonds. The second pathway involves indirect damage via free radicals formed after breakage of covalent bonds. Viruses appear to be inactivated primarily by direct damage, via disruption of the genome [95].

This inactivation procedure has been investigated for FMDV, Rauscher leukemia virus, HSV [96] and Lassa virus (Table 8) [97]. Inactivation kinetics showed that Rauscher leukemia virus and HSV were inactivated at a dose of 25 kGy. FMDV was inactivated with a dose of 40 kGy, and this was used successfully as antigen in the preparation of a vaccine against FMDV. The vaccine effectively protected the inoculated cattle against the disease [96]. In another study, rhesus monkeys were vaccinated with gamma irradiation-inactivated Lassa virus. The vaccinated animals had antibodies against the three major viral proteins. However, after challenge all the monkeys showed viremia and died. The inactivated Lassa virus vaccine based on gamma irradiation could not protect the animals after challenge [97].

Precaution is necessary when it comes to the preservation of the viral structure on inactivation by gamma irradiation. Gamma irradiation could inactivate PRRSV with the preservation of the viral entry-associated domains [41], but for other viruses the formation of free radicals should be investigated, as they might destroy the viral proteins [95].

In conclusion, gamma irradiation seems to inactivate viruses properly. Some KV vaccines based on gamma irradiation inactivation protect the host against disease after challenge with virulent virus, whereas others cannot prevent viremia and disease. This can be due to the formation of free radicals, which destroy viral proteins.

## Expert commentary

Some issues that are important for virus inactivation for vaccine purposes have been defined. A crucial step in the development of a KV vaccine is a balanced inactivation of the virus. All virus particles need to be completely inactivated, but the inactivation

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**Table 7. Effect of denaturing agents on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs).**

| Study (year) | Genome | Envelope | Capsid | Virus Family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| | DNA (ss) | No | I | Parvo | No | | | | | | | |
| | | | | Circo | No | | | | | | | |
| | DNA (ds) | Yes | I | Hepadna | No | | | | | | | |
| | | | | Herpes | No | | | | | | | |
| | | | | Asfor | No | | | | | | | |
| | | | C | Pox | No | | | | | | | |
| | | No | I | Adeno | No | | | | | | | |
| | | | | Papova | No | | | | | | | |
| | RNA (segmented, ss, -) | Yes | H | Arena | No | | | | | | | |
| | | | | Bunya | No | | | | | | | |
| Jonges *et al.* (2010) | | | | Orthomyxo | Influenza | Yes (>55°C) | Yes | NT | NT | NT | NT | [62] |
| | RNA (nonsegmented, ss, -) | Yes | H | Filo | No | | | | | | | |
| | | | | Paramyxo | No | | | | | | | |
| Anderson *et al.* (2008) | | | | Rhabdo | HNV | Yes (50°C) | NT | NT | NT | NT | No | [67] |
| | | | | Borna | No | | | | | | | |
| | RNA (nonsegmented, ss, +) | Yes | I | Toga | No | | | | | | | |
| | | | | | No | | | | | | | |
| Delrue *et al.* (2009) | | | | Arteri | PRRSV | Yes (pH2 or 12) | Yes | NT | NT | NT | NT | [41] |
| Delrue *et al.* (2009) | | | | | | Yes (37°C) | No | NT | NT | NT | NT | [41] |
| Poon *et al.* (2005) | | | | Retro | HIV | Yes (62°C) | NT | Yes | Yes | NT | NT | [87] |
| | | | | Flavi | No | | | | | | | |

+: Positive stranded; -: Negative stranded; C: Complex; ds: Double stranded; H: Helical; HNV: Hematopoietic necrosis virus; I: Icosahedral; NT: Not tested; PRRSV: Porcine reproductive and respiratory syndrome virus; SARS-CoV: Severe acute respiratory syndrome virus; ss: Single stranded; VN Ab: Virus-neutralizing antibodies; VS Ab: Virus-specific antibodies.

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**Table 7. Effect of denaturing agents on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs) (cont.).**

| Study (year) | Genome | Envelope | Capsid | Virus Family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| Darnell *et al.* (2004) | | | H | Corona | SARS-CoV | Yes (pH1–3 or 12–14) | NT | NT | NT | NT | NT | [30] |
| Darnell *et al.* (2004) | | | | | | Incomplete (56, 65 and 75°C) | NT | NT | NT | NT | NT | [30] |
| | | No | I | Calici | No | | | | | | | |
| | | | | Picorna | No | | | | | | | |
| | | | | | No | | | | | | | |
| | | | | Astro | No | | | | | | | |
| | RNA (segmented, ds) | No | I | Reo | No | | | | | | | |
| | | | | Birna | No | | | | | | | |

+: Positive stranded; -: Negative stranded; C: Complex; ds: Double stranded; H: Helical; HNV: Hematopoietic necrosis virus; I: Icosahedral; NT: Not tested; PRRSV: Porcine reproductive and respiratory syndrome virus; SARS-CoV: Severe acute respiratory syndrome virus; ss: Single stranded; VN Ab: Virus-neutralizing antibodies; VS Ab: Virus-specific antibodies.

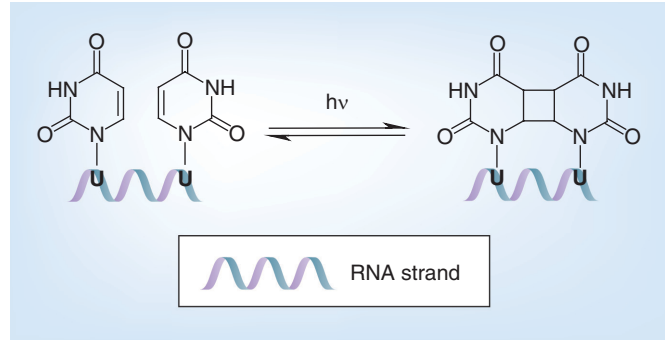


**Figure 7. Ultraviolet irradiation results in pyrimidine dimer formation.**
U: Uracil.

method should only have a minor effect on the viral antigenic properties, as the immune system of the host has to recognize the neutralizing epitopes to produce neutralizing antibodies against the antigen. As the antibody response is mostly investigated in vaccination studies, this review focused on the induction of antibodies on vaccination. However, the effect on cell-mediated immune response is also of crucial importance and should be investigated in more depth when evaluating candidate vaccines. The virus inactivation as well as the virus structure preservation can be a problem that should be kept in mind when developing a KV vaccine.

First, during the inactivation process one should be aware of possible virus aggregate formation, which can lead to viruses escaping inactivation. A single virus titration does not always detect escaping virus. A quality control system to investigate the safety of an inactivated virus vaccine can be obtained by passaging the treated virus *in vitro*, which can give more information about the completeness of the virus inactivation. Only if the vaccine passes such a test, can further animal experiments be considered to confirm vaccine safety.

Second, in the development of a KV vaccine, the preservation of the virus structure is very important. Inactivation procedures acting on viral proteins, such as cross-linkers or denaturing procedures [24,39,47,80,82,83], may modify the viral-neutralizing epitopes that are essential for induction of neutralizing antibodies. Thus, it is better to inactivate the virus for KV vaccines with methods that do not affect the viral proteins. Inactivation procedures mainly acting on the genome, such as radiation and alkylating agents [72,90,95], will most likely preserve viral-neutralizing epitopes and should be preferred. Nevertheless, protein degradation after inactivation should always be checked.

Different quality control systems to investigate the antigen quality are available. For influenza, the effect of inactivation is investigated by measuring the hemagglutinating activity before and after inactivation [98]. ELISA may be used for the determination of the attachment of neutralizing antibodies to viral epitopes after inactivation. This is used as quality control of the antigens of HIV [87,99], rabies virus [100,101] and PV vaccines [102]. However, for the previous methods, knowledge of the neutralizing epitopes is necessary. Sometimes, the neutralizing epitopes are not known. As

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**Table 8. Effect of irradiation on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs).**

| Study (year) | Genome | Envelope | Capsid | Virus Family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| | DNA (ss) | No | I | Parvo | No | | | | | | | |
| | | | | Circo | No | | | | | | | |
| | DNA (ds) | Yes | I | Hepadna | No | | | | | | | |
| Smolko *et al.* (2005) | | | | Herpes | HSV | Yes (25 kGy) | NT | NT | NT | NT | NT | [96] |
| | | | | Asfor | No | | | | | | | |
| | | | C | Pox | No | | | | | | | |
| | | No | I | Adeno | No | | | | | | | |
| | | | | Papova | No | | | | | | | |
| McCormick *et al.* (1992) | RNA (segmented, ss, -) | Yes | H | Arena | Lassa | Yes (gamma) | NT | Yes | NT | No | No | [97] |
| | | | | Bunya | No | | | | | | | |
| | | | | Orthomyxo | No | | | | | | | |
| | RNA (nonsegmented, ss, -) | Yes | H | Filo | No | | | | | | | |
| | | | | Paramyxo | No | | | | | | | |
| | | | | Rhabdo | No | | | | | | | |
| | | | | Borna | No | | | | | | | |
| | RNA (nonsegmented, ss, +) | Yes | I | Toga | No | | | | | | | |
| | | | | | No | | | | | | | |

+: Positive stranded; -: Negative stranded; C: Complex; ds: Double stranded; FMDV: Foot-and-mouth disease virus; H: Helical; I: Icosahedral; MLV: Murine leukemia virus; NT: Not tested; PRRSV: Porcine reproductive and respiratory syndrome virus; RHDV: Rabbit hemorrhagic disease virus; RLV: Rauscher leukemia virus; ss: Single stranded; VN Ab: Virus-neutralizing antibodies; VS Ab: Virus-specific antibodies.

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**Table 8. Effect of irradiation on different viruses: inactivation, viral protein modification, induction of virus-specific and virus-neutralization antibodies and protection on challenge (reduction of viremia and fewer clinical signs) (cont.).**

| Study (year) | Genome | Envelope | Capsid | Virus Family | Example | Inactivation (concentration) | Viral protein modification | Induction | | Protection on challenge | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *VS Ab* | *VN Ab* | *Reduced viremia* | *Fewer clinical signs* | |
| Delrue *et al.* (2009) | | | | Arteri | PRRSV | Yes (0.25 kGy) | No | NT | NT | NT | NT | [41] |
| Delrue *et al.* (2009); Vanhee *et al.* (2009) | | | | | | Yes (100 mJ/cm$^2$) | No | Yes | Yes | Yes | NT | [41,79] |
| Sarzotti *et al.* (1994) | | | | Retro | MLV | Yes (120 ergs/s/mm$^2$) | NT | NT | NT | Yes | Yes | [93] |
| Smolko *et al.* (2005) | | | | | RLV | Yes (25 kGy) | NT | NT | NT | NT | NT | [96] |
| | | | | Flavi | No | | | | | | | |
| | | | H | Corona | No | | | | | | | |
| Henning *et al.* (2005) | | No | I | Calici | RHDV | Yes (0.0078 W/cm$^2$) | NT | No | No | No | No | [92] |
| Smolko *et al.* (2005) | | | | Picorna | FMDV | Yes (40 kGy) | NT | NT | NT | NT | Yes | [96] |
| Miller *et al.* (1974) | | | | | Mengovirus | Yes (7 kergs/min/mm$^2$) | Yes | NT | NT | NT | NT | [90] |
| | | | | | No | | | | | | | |
| | | | | Astro | No | | | | | | | |
| Subasinghe *et al.* (1972) | RNA segmented, ds) | No | I | Reo | Reovirus | Yes (ultraviolet) | Yes | NT | NT | NT | NT | [94] |
| | | | | Birna | No | | | | | | | |

+: Positive stranded; -: Negative stranded; C: Complex; ds: Double stranded; FMDV: Foot-and-mouth disease virus; H: Helical; I: Icosahedral; MLV: Murine leukemia virus; NT: Not tested; PRRSV: Porcine reproductive and respiratory syndrome virus; RHDV: Rabbit hemorrhagic disease virus; RLV: Rauscher leukemia virus; ss: Single stranded; VN Ab: Virus-neutralizing antibodies; VS Ab: Virus-specific antibodies.

virus-neutralizing antibodies are often directed to viral proteins involved in virus entry [41,103–105], it can be advised to preserve virus entry-associated domains to induce a virus-neutralizing antibody response. In this context, a quality control system for investigating the antigen quality of the inactivated virus can be developed by confirming the preservation of entry-associated domains of a virus [41]. This can be achieved by investigating attachment and internalization of virus into the host cells before and after virus inactivation by immunofluorescence staining and confocal microscopy [41].

In conclusion, many methods have been developed that are potentially suitable to inactivate a certain vaccine virus, but the success of these methods may vary depending on the particular virus and the conditions used (i.e., temperature, pH and concentration). Nonetheless, successful methods to inactivate a virus for vaccine development are best chosen from methods that mainly have an effect on the genome. Since formaldehyde, glutaraldehyde, pH and heat have an influence on protein level, it is possible that the neutralizing epitopes are modified by cross-linking (formaldehyde or glutaraldehyde) or denaturation (pH or temperature). In our opinion, UV irradiation, gamma irradiation, BPL and BEI are the most successful methods to inactivate a virus to create a KV vaccine, as these inactivation procedures mainly target the genome and have no or minor effects on viral proteins. However, it has to be considered that viral proteins may be cross-linked by BPL, or damaged by UV photoproducts and free radicals formed by gamma irradiation. The radiation target theory, however, predicts that the radiation sensitivity of biomolecules depends on their mass [106]. Therefore, viral genomes, with a significantly lower mass, would be significantly more sensitive to damage than proteins.

## Five-year view

Until recently, the search for an effective KV vaccine was often a process of trial and error, in which a virus was inactivated by a certain inactivation method and then immediately tested in animals to investigate complete virus inactivation, antibody response on vaccination and protection on challenge. Currently, a series of *in vitro* tools is available, allowing the creation of a quality control system, in which virus inactivation (safety) and antigen quality (immune response) can be investigated. In this way, vaccine efficacy can be predicted prior to animal experiments. As a consequence, outbreaks due to incomplete virus inactivation and poor immune responses due to viral protein destruction can be avoided as much as possible.

It appears that virus inactivation methods exerting their main effects on the viral genome have most potential in generating an effective immune response and protecting the host against disease. On *in vitro* testing of several inactivation methods following a quality control system for an inactivated PRRSV vaccine, we have observed that only a few of the available methods allow complete virus inactivation with preservation of the virion structure. Considering the molecular and mechanistic basis of these evaluated methods, it is clear that they all mainly affect the viral genome, leaving the viral proteins unaffected. In our opinion, it is therefore of crucial importance for the induction of a proper immune response and protection against disease, to evolve the development methods for KV vaccines based on viral genome inactivation, which can guarantee an intact virion structure.

**Financial & competing interests disclosure**

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*No writing assistance was utilized in the production of this manuscript*

## Key issues

- For safety reasons, killed virus (KV) vaccines are preferred over modified live virus vaccines. However, KV vaccines can suffer from problems related to efficacy.
- A good *in vitro* quality control system is required for prediction of the effectiveness of a KV vaccine. This quality control system should allow verification of the completeness of virus inactivation and the quality of the resulting antigen.
- In order to create a safe KV vaccine, the virus inactivation method should inactivate the virus mixture completely without risk of resistant virus particles. Due to aggregate formation, incompletely inactivated virus particles can still be present in the vaccine with outbreaks as a consequence.
- For the generation of a KV vaccine able to induce a proper immune response, antigen quality is of high importance. Due to protein crosslinking, protein denaturation and protein degradation, the viral antigen can be completely destroyed, with a poor immune response as a consequence.
- Crosslinkers, such as formaldehyde and glutaraldehyde, or denaturing processes, such as pH and heat, can have an influence on viral proteins and destroy the virion structure. As a consequence, KV vaccines based on these inactivation methods might not induce a proper immune response.
- Next to alkylating agents, such as β-propiolactone and binary ethylene imine, ultraviolet and gamma irradiation mainly influence the viral genome, preserving the virion structure. These methods are thus highly recommended for generation of an efficient and safe KV vaccine.

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