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# INACTIVATION OF PICORNAVIRUSES AND CALICIVIRUSES; Part 2: Inactivation

Suphachai Nuanualsuwan<sup>1\*</sup> Dean O. Cliver<sup>2</sup>

## Abstract

Suphachai Nuanualsuwan<sup>1\*</sup> Dean O. Cliver<sup>2</sup>

## INACTIVATION OF PICORNAVIRUSES AND CALICIVIRUSES; Part 2: Inactivation

The prevention of human fecal contamination of food and water is an effective measure to control the transmission of foodborne disease viruses e.g. hepatitis A virus (HAV) and calicivirus found in food chains and water systems. If the prevention of virus contamination is not successful, one of the most common critical control points in the food industry is the inactivation of viruses that contaminate the food or water. The objective of inactivation is to modify the virus or make it abort in the next replicative cycle. The efficiency of inactivating agents is measured by the plots of "logarithmic scale of plaque forming units of virus" versus the "inactivating dose". The slope of the best-fit straight line is the rate of inactivation by a specific agent. Ultraviolet (UV) light and hypochlorite are used to disinfect water, food surfaces and food-contact surfaces. The most common inactivation method in food preparation and processing is heat. The virucidal wavelength of UV is 253.7 nanometers and the decimal inactivation doses of viruses are between 1-25 mWs/cm<sup>2</sup>. Depending on pH, hypochlorous acid (HOCl) inactivates viruses more effectively than the hypochlorite ion (OCl<sup>-</sup>). Thermal treatment inactivates virus at a temperature above 60°C in minutes. Some gram-positive bacteria e.g. *Micrococcus luteus* and *Staphylococcus epidermidis* enzymatically inactivate enteroviruses. HAV and feline calicivirus appears to be more resistant to inactivation than other picornaviruses and coliphages. Inactivation methods have different mechanisms of action in which the target is not mutually exclusive and may be controversial. In essence, the viral capsid is the primary target of chlorine, thermal, biodegradation, drying and formaldehyde inactivation while the primary target of UV inactivation is the viral RNA.

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**Keywords :** Inactivation, Foodborne disease, Picornaviruses, Caliciviruses

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## บทคัดย่อ

ศุภชัย เนื่อนवलสุวรรณ<sup>1\*</sup> Dean O. Cliver<sup>2</sup>

### การทำลายไวรัสพิกอร์น่า (Picornaviruses) และไวรัสแคลิซิ (Caliciviruses) (ตอนที่ 2 การทำลายไวรัส)

วิธีการหนึ่งที่สามารถควบคุมการแพร่ไวรัสที่ก่อโรคโดยผ่านอาหาร เช่น ไวรัสตับอักเสบ และ ไวรัสแคลิซิ คือ การป้องกันมิให้มีการปนเปื้อนของอุจจาระคนเข้าไปสู่ห่วงโซ่อาหารและระบบหมุนเวียนน้ำ ถ้าไม่สามารถป้องกันการปนเปื้อนได้ วิธีอื่นที่สามารถควบคุมการแพร่ไวรัส คือ การทำลายเชื้อที่ปนเปื้อนอยู่ในอาหารและน้ำอยู่แล้วซึ่งถือว่าเป็นจุดควบคุมวิกฤติหนึ่งที่สำคัญในขบวนการผลิตอาหาร วัตถุประสงค์หลักในการทำลายเชื้อคือการเปลี่ยนแปลงไวรัสหรือทำให้ไวรัสไม่สามารถเพิ่มจำนวนได้ การประเมินประสิทธิภาพของวิธีการทำลายไวรัสโดยการเปรียบเทียบความชัน (slope) ของกราฟความสัมพันธ์ระหว่างจำนวนไวรัสที่ถูกทำลายกับระดับการทำลาย แสงอัลตราไวโอเล็ต (ultraviolet light: UV) หรือคลอรีนใช้สำหรับการทำลายไวรัสในน้ำ ผิวน้ำ และบริเวณพื้นผิวสัมผัสอาหาร ความร้อนเป็นวิธีการทำลายเชื้อไวรัสที่นิยมใช้มากที่สุดในขบวนการผลิตอาหาร ความถี่ของ UV ที่สามารถทำลายไวรัสได้ คือ 253.7 นาโนเมตร และปริมาณแสงที่สามารถทำลายไวรัสให้เหลือร้อยละ 10 ของไวรัสเริ่มต้นจะมีค่าอยู่ระหว่าง 1-25 มิลลิวัตต์วินาทีต่อตารางเซนติเมตร กรดไฮโปคลอไรต์มีประสิทธิภาพในการทำลายไวรัสได้ดีกว่าประจุไฮโปคลอไรต์ โดยขึ้นอยู่กับความเป็นกรดต่าง(pH)ด้วย ความร้อนที่อุณหภูมิอย่างน้อย 60°C สามารถทำลายไวรัสได้ภายในเวลาไม่กี่นาที แบคทีเรียแกรมบวก เช่น *Micrococcus luteus* และ *Staphylococcus epidermidis* ทำลายไวรัสโดยอาศัยเอนไซม์ ไวรัสตับอักเสบเอและไวรัสแคลิซิแมว(feline calicivirus)ดูเหมือนว่าจะทนต่อการทำลายเชื้อมากกว่าไวรัสลำไส้ชนิดอื่น ๆ และโคไลฟาจ กลไกในการทำลายไวรัสมีความแตกต่างกัน ซึ่งวิธีการทำลายไวรัสมักจะไม่สามารถทำลายเฉพาะส่วนประกอบหนึ่งของไวรัสโดยตรงเท่านั้นและบางครั้งกลไกการทำลายก็ยังเป็นที่โต้แย้งกันอยู่ พบว่าเป้าหมายที่สำคัญในการทำลายไวรัสโดยคลอรีน ความร้อน จุลชีพ ความแห้ง และฟอร์มอลดีไฮด์ อยู่ที่แคปซิดของไวรัส แต่โดยแสง UV อยู่ที่สายพันธุกรรม RNA

คำสำคัญ: ชีววิทยา การทำลายเชื้อ ไวรัสพิกอร์น่า ไวรัสแคลิซิ

#### 4. Inactivation activity

Given the fact that virus particles in their transmissible form, cannot multiply or propagate outside specific host cells (extracellularly), the measures needed to control transmission appear to be easy and uncomplicated (Cliver, 1997). However, viral foodborne outbreaks, particularly those from gastroenteritis viruses, have increased at an alarming rate in the past few years (CDC, 1999, 2001). This suggests that virus foodborne diseases are more complicated and prevalent than bacterial foodborne diseases (Mead et al., 1999). The ultimate goal of prevention is to avoid human fecal contamination in food chains and water systems.

When only a few virus particles get inside the human host, replicative cycles result in exponential numbers of progeny virus particles in the feces (Racaniello, 2001). Food handlers without proper sanitation can readily and unknowingly spread the virus to consumers and the public via fecally contaminated hands (Cliver, 1997). Therefore, in the food industry, dealing with viruses transmitted by the fecal-oral route, good personal hygiene practices and high standards of food protection and sanitation are extremely critical, in order to protect consumers from viral infections. Adequate hand-washing facilities, proper hand-washing techniques, gloves (Ansari et al., 1989), and immunization against specific virus

infections (WHO, 1995) should be taken into account to achieve good food safety.

If the prevention of virus contamination of food or water is not successful, critical steps must be incorporated into the processing of food in order to get rid of these causative agents and ensure high safety standards (Cliver, 1997). One of the most common critical control points (CCP) for the food industry is the inactivation of the viruses contaminating the food or water (Cliver, 1997). Inactivation of the virus means the permanent loss of infectivity, which is the ability of viruses to make people sick (Russell et al., 1999). The most common method of inactivation preparation and processing of food and in emergencies, with water, is heat. Ultraviolet (UV) light and hypochlorite is normally used to disinfect water, as well as food surfaces and food-contact surfaces.

In order to evaluate virus inactivation properly, virus quantitation needs to be standardized, reproducible and specific (Russell et al., 1999). Since the viruses of interest are human pathogens, the infectivity assay perfectly fits the criteria above. Quantal assay examines the noticeable outcome of dilution of the virus suspension e.g., death of the experimental animals, cytopathic effect (CPE) or death of the cell culture. The end-point of a quantal titration is the dilution at which the virus infects or kills 50% of inoculated hosts when the infectious units can be called an infectious dose ( $ID_{50}$ ) for experimental animals or  $TCID_{50}$  for tissue cultures (Hsiung and Fong, 1982). Production of lesions on the chorio-allantoic membrane of embryonic eggs is also used to calculate the concentration of infectious or viable virions. The most probable number (MPN) method is also used to statistically estimate the virus titer. *Plaque assay* entails a serial dilution of the virus suspension, which is inoculated onto confluent monolayers of a specific host-cell culture. After the adsorption or attachment steps, the monolayer is overlaid with fluid medium plus agarose, to restrict the progeny virus movement in the vicinity of infected host cells. As each virion ideally makes one plaque (analogous

to a bacterial colony), a localized focus of infected and dead cells is grossly observable, after the cell monolayers are stained with dye e.g. 0.5% crystal violet. The infectivity titer of the original suspension is recorded as plaque-forming units (PFU/ml) (Hsiung and Fong, 1982; Nuanualsuwan and Cliver, 2002<sup>a</sup>).

Inactivation tests are commonly divided into four methods (Russell et al., 1999). *Carrier* methods are useful for testing disinfectants intended for use on a dry surface. Disinfectants are agents, typically chemical, which get rid of pathogenic microorganisms when applied to an inanimate object (Thurman and Gerba, 1988). The test virus suspension is inoculated onto a hard, nonporous surface, permitted to dry and then inactivated with serial dilutions of disinfectant. *Plaque-suppression* methods: the cell culture monolayers are inoculated with virus and then a filter paper soaked with the disinfectant is applied onto the monolayer. After incubation, the discs are removed and visualized for the survival of cell cultures by dye staining. *Suspension* methods apply a mixture of virus suspension and disinfectant. The mixture is usually incubated at a certain temperature for fixed intervals and the residual infectivity of the virus is determined in cell culture by plaque assay. The test results are reported as a  $\log_{10}$  titer reduction. Virus aggregate formation is sometimes a problem in inactivation testing; aggregates can be the result of virus association with host-cell debris or can be disinfectant-induced (precipitated protein). Aggregates of above 100 infectious particles, which are not unusual for enterovirus preparations, may appear as only one individual infectious unit after a  $2 \log_{10}$  infectivity titer reduction (Moldenhauer, 1984). *Bacteriophage* methods use bacterial lawns on petri plates as hosts, instead of monolayers of mammalian cell cultures and measure the infectivity in a similar way to plaque assay (Russell et al., 1999).

#### 4.1 Inactivation kinetics

Bacterial inactivation is easier to accomplish than virus inactivation (Thurman and Gerba, 1988). The simplicity and the lack of an extracellular metabolism

make viruses more resistant than bacteria to many treatments (Poduska and Hershey, 1972). The objective of inactivation is to modify the virus or make it abort in the next replicative cycle. Therefore, the intervention step to block the replicative cycle can involve immobilizing the viruses on a surface, blocking or destroying the receptor-attachment site on the virus, or damaging the nucleic acid inside the virus capsid (Thurman and Gerba, 1988). The factors that affect inactivation efficiency can be the nature and the state (clumping or aggregation) of the virus (Jensen et al., 1980), the available concentration of the inactivating agent (Varma et al., 1974; Engelbrecht et al., 1980; Taylor and Butler, 1982), exposure time, pH, temperature, any interfering substances and the level of infectivity titer (Engelbrecht, 1978; Sharp and Leong, 1980; Grabow et al., 1983; Akey and Walton, 1985; Keswick et al., 1985; Payment et al., 1985).

Inactivating agents can be soluble, insoluble (contact) and light-induced (Thurman and Gerba, 1988). *Soluble* inactivating agents need to be monitored and replenished from time to time because of their short half-life in water systems. However in the case of chlorine it is more practical to adjust the concentration depending on the chlorine demands of water treatment and then again when the concentration decreases to below the optimal level, as diffusion or dilution takes place. *Insoluble* or surface-active inactivating agents require physical contact with the virus over their surface, by physisorption or chemisorption e.g., metal and organic substances (Thurman and Gerba, 1988). *Light-induced* inactivations can be ultraviolet light (UV) or photodynamic. Photodynamic inactivation is the inactivating sensitization of microorganisms by a dye when the microorganism is exposed to visible light e.g. poliovirus is inactivated by methylene blue, in conjunction with visible light (Thurman and Gerba, 1988).

Inactivation kinetics can be compared to a chemical reaction (Thurman and Gerba, 1988). In order to achieve high reproducibility and ideal inactivation, the following areas should be emphasized. The separate and

individual virion particles are uniformly susceptible to the inactivating agents (no subpopulations). The reaction and doses of inactivating agents are essentially constant. Both viruses and inactivating agents are homogeneously brought together in the reaction environment. Clearly, the main emphasis is the even mixture of discrete units of virion particle and inactivating agents. The efficiency of inactivating agents is measured by the plots of "logarithmic scale of plaque forming units of virus" on the Y-axis versus "inactivating dose" on the X-axis. The best-fit, straight-line curve represents exponential and efficient inactivation. Thus, the inactivation can be considered to have "*one-hit kinetics*" or "*first-order kinetics*." The inverse of the slope of this straight line is the rate of inactivation of virus by a specific agent.

Deviations of inactivation from linearity happens in various ways (Thurman and Gerba, 1988). The improper mixing of viruses and the inactivating agent or slow penetration of inactivating agents into the target site (e.g. nucleic acid inside the virus capsid) can result in a *shoulder effect*, since the inactivating agents have to penetrate the virus capsid before attacking the target nucleic acid. Additionally, the shoulder effect happens in multihit kinetics where the target sites of the virus, require multiple hits before being inactivated or when the virus clumps or aggregates. The presence of a highly susceptible subpopulation in the virus suspension can result in a *rapid initial inactivation*. *Tailing-off* happens when the concentration of inactivating agents diminishes, or in the presence of a more resistant subpopulation, as a result of cell-associated clumping protection, conformational change of capsid, or genetic drift or shift take place (Hoff and Water Engineering Research Laboratory, 1986). In cases where the infectivity titer of the virus suspension is initially high and residual infectivity is not allowed, the inactivating agent may have to attack the target more than once (even though it is known that one target hit is enough to inactivate that virion particle), since the high titer demands a higher dose of inactivating agent. This phenomenon is sometimes called *redundancy* of inactivation.

#### 4.2 Mechanisms of inactivation

The target inactivating agents seek on virus particles, can be the viral envelope, the capsid, or its nucleic acid and the inactivation can be permanent or reversible (Thurman and Gerba, 1988; Russell et al., 1999). Inactivating agents can modify the functional structures of the virus capsid that are responsible for the extra-cellular phase (protecting virus nucleic acid) and the early stages of the replicative cycle in the host cell e.g., receptor-attachment sites reacting with cell receptors and affecting capsid integrity, capsid conformation or capsid permeability. The receptor-attachment site needs the correct amino acid sequences and unaltered conformation to accomplish the attachment of virus to the host cell receptor. When the capsid permeability or structure is compromised, the susceptible nucleic acid is released and no longer enclosed by the capsid and can be digested by ribonuclease (RNase), which is outside or penetrating through the now permeable capsid. For enteroviruses and caliciviruses, the viral genome per se is infectious if it somehow can get inside the host cell. Theoretically, virus inactivation is accomplished only when the viral genome is essentially inactivated.

#### 4.3 Ultraviolet inactivation

Ultraviolet (UV) radiation has been used to disinfect public water supplies (Thurman and Gerba, 1988). UV radiation energy waves (between visible light and x-ray) are in the range of electromagnetic waves of 100 to 400 nanometers (nm). UV radiation may be categorized as vacuum UV (100-200 nm), UVC (200-280 nm), UVB (280-315 nm), and UVA (315-400). The optimal inactivating range is between 245-285 nm. A medium pressure lamp emits UV between 180-1370 nm whereas a low-pressure mercury-vapor discharge lamp or germicidal lamp with a tubular glass envelope, emits short-wavelength UV radiation with the peak (monochromatic) at 253.7 nm and containing only about 1% of other wavelengths. The relative dose of ultraviolet radiation is measured from a source of constant UV radiation within a set period of time, depending on the specific conditions of the experimental

setup. The UV dose is defined and is usually measured as incident energy (not absorbed energy), which is the product of constant UV intensity or dose rate in units of mW/cm<sup>2</sup> and time in units of seconds. The exponential pattern of UV inactivation can be called one-hit kinetics since only a single UV photon, absorbed and hitting the critical target of a virus particle, is required to inactivate that virus particle. The inverse of the inactivation rate (slope of best-fit straight-line curve) is defined as the UV dose that is required to inactivate 90% of the viruses (one log<sub>10</sub> inactivation), the so-called decimal inactivation dose (DID); it is analogous to the D value (decimal reduction time or DRT) or time that is required to inactivate 1 log of virus infectivity in thermal inactivation procedures.

In contrast to most (chemical) disinfectants, UV radiation effectively and rapidly inactivates pathogens, by the transfer of the electromagnetic energy of a mercury arc lamp, through a photochemical reaction with virus nucleic acids (Jagger, 1967). This makes it unnecessary to generate, handle, transport, or keep corrosive and hazardous chemical reagents on the premises. Furthermore, UV disinfection equipment requires less space than other disinfection methods. UV radiation has been widely used to disinfect (ground) water and wastewater (Slade et al., 1986; Loge et al., 1996;) and has been measured against hepatitis A virus (HAV) (Wang et al., 1995), poliovirus (Wetz et al., 1983), and MS2 and øx174 (Battigelli et al., 1993) coliphages (viruses that infect the bacterium, *Escherichia coli*), but not against feline calicivirus (FCV) nor Noroviruses (NLVs). Coliphages have been used as surrogates for animal viruses in general, because the coliphages can be assayed more rapidly and cheaply (Mariam and Cliver, 2000). The DID of HAV, poliovirus (PV), MS2 and øx174 coliphages are ca. 5-36 mWs/cm<sup>2</sup> (Wiedenmann et al., 1993; Wang et al., 1995; Nuanualsuwan et al., 2002), 6-25 mWs/cm<sup>2</sup> (Chang et al., 1985; Abbaszadegan, 1993; Nuanualsuwan et al., 2002), 18-35 mWs/cm<sup>2</sup> (Battigelli et al., 1993; Wiedenmann et al., 1993; Nuanualsuwan et al., 2002) and 1-15 mWs/cm<sup>2</sup> (Battigelli et al., 1993; Nuanualsuwan et al., 2002),

respectively.

UV affects both the viral capsid and its nucleic acid with different degrees of significance (Jagger, 1967). Nucleic acid is 10 times more sensitive to UV than are the proteins or amino acids, because all nucleic acid bases absorb UV very well and rarely have a tertiary structure. However, the sugar and phosphate groups do not absorb UV well, so the significant sites of action are the bases of the nucleic acid. Among all bases, pyrimidine bases are 10 times better UV absorbers than are purine bases. The major photoproducts are photohydrates and photodimers. The common photoproducts are uridine hydrate, uracil dimer, cytidine hydrate, cytidilic hydrate and thymine dimers. Among amino acids, the aromatic amino acids e.g. tyrosine, tryptophan, and phenylalanine are more sensitive to UV and may undergo decarboxylation, deamination, or ring rupture (Thurman and Gerba, 1988), causing overall conformational changes that can lead to a loss of function of the viral capsid.

During inactivation of PV-1 by UV, the infectivity of PV-1 was compared with that of its RNA. Diethylaminoethyl (DEAE)-dextran (1 mg/ml in Dulbecco's Modified Eagle Medium buffered with 0.05 M Tris, pH 7.4) was used to facilitate PV-1 RNA into FRhK-4 host cells. The slopes of best-fit regression lines of inactivation curves for virion infectivity and RNA infectivity, were compared, to determine the target of inactivation. The slopes of the inactivation curves of virion infectivity and RNA infectivity were not statistically different ( $p > 0.1$ ). So the target of UV inactivations is viral RNA (Nuanualsuwan and Cliver, 2002<sup>b</sup>).

The viral capsid integrity viruses were also tested for their functional structures after being inactivated with UV (Table 1) (Nuanualsuwan and Cliver, 2002<sup>a,c</sup>). The capsid of HAV, PV-1, FCV inactivated with UV protected the viral RNA against ribonuclease (RNase). However, the capsid could not protect the viral RNA against RNase and protease when applied simultaneously. When PV-1 was inactivated by UV, the ability of UV-

inactivated PV-1 to attach to cell receptors and to react with the homologous antibody, was lost. On the other hand, the two functional structures of UV-inactivated HAV and FCV were retained. So the primary target of UV inactivation is mainly the viral RNA but also to a certain extent the viral capsid

#### 4.4 Chlorine inactivation

Chlorine is widely used for general-purpose household disinfection, water and wastewater disinfection, general sanitation in food services and food manufacturing and also is often recommended as the standard disinfectant for the inactivation of viral pathogens because of its oxidizing, broad spectrum and residual activities (Thurman and Gerba, 1988; Russell et al., 1999). At a pH of approximately 7.0, the C x T (concentration x time) values (mg/L-min) to inactivate 90% of HAV, PV-1 and FCV are 7.0 (Sobsey et al., 1991), 0.717 (Poduska and Hershey, 1972; Scarpino et al., 1974) and 0.4 (Doultree et al., 1999), respectively. The dissociation of hypochlorous acid (HOCl) is dependent upon the pH and the equilibrium between HOCl and hypochlorite ion (OCl<sup>-</sup>). At a pH lower than 6.3, ca., 90% is in the form of HOCl and HOCl is more effective than OCl<sup>-</sup> (Lippy, 1986; Sletten, 1974; Koh et al., 1975).

The target of chlorine is very controversial, it is debatable whether the effect is on the viral capsid or the RNA (Thurman and Gerba, 1988). Effects on the viral capsid by chlorine, can be capsid protein denaturation and conformational or structural changes, without an effect on RNA infectivity (Chang, 1970; Tenno et al., 1979; Alvarez and O'Brien, 1982<sup>b</sup>; Young and Sharp, 1985). Additionally, the conformational changes of the capsid, which is associated with infectivity, is hypothetically reversible because the attachment of the virus to host cell receptors, is sensitive to a change of hydrophobicity (Shaffer et al., 1980; Snead et al., 1980; Payment et al., 1985; Rose et al., 1986). It was found that the ability of the capsid of hypochlorite-inactivated HAV, PV-1 and FCV to protect viral RNA against RNase and protease, was lost and so was any attachment to the cell receptors

(Table 2) (Nuanalsuwan and Cliver, 2002<sup>a,c</sup>). The reactivity of viral capsid of HAV and FCV (except PV-1) with corresponding homologous antibodies was lost when these viruses were inactivated with hypochlorite.

On the other hand, some studies show that nucleic acid is the target site of chlorine inactivation, as a result of cleavage and then the release of RNA, although but the pI and attachment of capsid are not compromised (Olivieri et al., 1971; Dennis, 1979; O'Brien and Newman, 1979; Alvarez and O'Brien, 1982<sup>a</sup>; Taylor and Butler, 1982; Nuanalsuwan and Cliver, 2002<sup>b</sup>). For PV-1, the slopes of the inactivation curves of virion infectivity and RNA infectivity were not statistically different, which means that the viral capsid did not contribute to the loss of either virion or RNA infectivity, being  $p > 0.1$  (Nuanalsuwan and Cliver, 2002<sup>b</sup>). The viral RNA of PV-1 and FCV inactivated by hypochlorite was not available for reverse transcription, polymerase chain reaction (RT-PCR), amplification (Table 2). However, some studies indicate that chlorine causes inactivation of both the viral capsid and RNA (O'Brien and Newman, 1979; Tenno et al., 1979; Alvarez and O'Brien, 1982<sup>b</sup>; Harakeh and Butler, 1984).

#### 4.5 Heat inactivation

Heat degradation destabilizes and disrupts the viral capsid of PV in a series of reactions leading to the breakdown of capsid and the release of RNA (Koch and Koch, 1985<sup>b</sup>). Virion disruption by heat depends on temperature, exposure time, ionic strength, etc. Heat inactivation also follows single hit kinetics.

Intact or native virions are characterized by their infectivity, the ability to attach to specific host cells, the D (N) antigenicity, a sedimentation coefficient of 150-160S in sucrose gradients, a buoyant density of 1.34 g/cm<sup>3</sup>, a pI of 7.0, a resistance to proteases and RNases, an impermeability to some dyes and a composition of 35S (viral RNA) plus 60 copies of viral proteins (VPs) (Koch and Koch, 1985<sup>a</sup>). With gentle to moderate heat (50-56°C), a native virion shows swelling (or breathing) of the viral capsid since the thermal energy loosens the bonding among VPs. The sedimentation coefficient

drops to 135S and buoyant density is unstable since the virion is fully hydrated. The swollen particles are reversible back to the native virion. With continued heat the swollen particles lose 60 copies of the VP4 simultaneously and become a 80-90S ribonucleoprotein particle (RNP) with pI at 4.5. The RNP is no longer infectious and fails to be adsorbed by cells. The loss of VP4 creates holes in the capsid of the RNP by which infectious RNA becomes sensitive to RNase. The sensitivity of infectious RNA is a result of the accessibility of RNase immigrating through open holes of the capsid or by protrusion of RNA from the capsid surface. This leaky capsid is characterized by sensitivity to protease and its permeability to the stains used for electron microscopy (such as phosphotungstic acid). The significant change of virion into RNP is the conversion of its antigenicity from D (N) to C (H). Following on from the formation of RNP is the release of infectious RNA from the capsid. This *empty* capsid has a stable buoyant density of 1.30 g/cm<sup>3</sup> and a  $S_{20w}$  of 73S. This shell is also called artificial top component (ATC) and is comparable to naturally-occurring top component (NTC). After losing copies of VP2, this empty capsid exists as a *skeleton* capsid having only VP1 and VP3.

After inactivation at 72°C to a stage when infectivity is not detected, the capsid of HAV, PV-1, and FCV is only slightly degraded. This degraded capsid could protect viral RNA against RNase but not against RNase and protease, when both were digesting the viral capsid at the same time (Table 3) (Nuanalsuwan and Cliver, 2002<sup>a</sup>). When inactivated at physiological temperatures (37°C), viral RNA protection against composite enzymes is not compromised (Table 4). The ability of HAV, PV-1, and FCV to attach to homologous host cell receptors was lost, regardless of the inactivating temperature, over the range of 37 to 72°C. On the other hand its reactivity with homologous antibody of 37°C- and 72°C-inactivated HAV and FCV, excepting PV-1, did not change. This resulted in a correlation between the loss of infectivity and changes of functional structure. The most



**Table 1.** Summary of disruption of viral capsid by ultraviolet inactivation

Virus	Viral capsid integrity			
	RNase protection		Attachment to cell receptors	Antigen reaction with antibody
	Without protease	With protease		
HAV	+ <sup>a</sup>	- <sup>b</sup>	+ <sup>c</sup>	+
PV-1 <sup>d</sup>	+	-	-	-
FCV	±	-	±	+

<sup>a</sup>Viral capsid function was retained

<sup>b</sup>Viral capsid function was lost

<sup>c</sup>Viral capsid function was marginal

<sup>d</sup>Viral RNA was found to have lost its infectivity

**Table 2.** Summary of disruption of viral capsid by hypochlorite inactivation

Virus	Viral capsid integrity			
	RNase protection		Attachment to cell receptors	Antigen reaction with antibody
	Without protease	With protease		
HAV	+ <sup>a</sup>	- <sup>b</sup>	-	+
PV-1 <sup>c</sup>	-	-	-	-
FCV	-	-	-	+

<sup>a</sup>Viral capsid function was retained

<sup>b</sup>Viral capsid function was lost

<sup>c</sup>Viral RNA was found to have lost its infectivity

sensitive feature of the inactivation process, appears to be the ability of the virus to attach to the homologous cell receptors.

When the slopes of the inactivation curves of virion infectivity and RNA infectivity of PV-1 were analyzed, the difference in the slopes of the inactivation curves was statistically significant for 72°C inactivation ( $0.01 < p < 0.025$ ) (Nuanualsuwan and Cliver, 2002<sup>b</sup>). It can be concluded that the 72°C inactivation did not cause the loss

of PV-1 RNA infectivity and that the target of high-temperature inactivation upon PV-1 is the capsid, not the RNA. The result is consistent with previous studies (Breindl, 1971; Rombaut et al., 1994), showing that heat inactivation caused conformational changes of capsid, which led to a loss of infectivity and antigenicity.

HAV is more heat stable than FCV (Provost et al., 1975; Parry and Mortimer, 1984; Siegl et al., 1984; Slomka and Appleton, 1998). The integrity and antigenicity of

**Table 3.** Summary of disruption of viral capsid and RNA at 72°C inactivation

Virus	Viral capsid integrity			Antigen reaction with antibody
	RNase protection		Attachment to cell receptors	
	Without protease	With protease		
HAV	+ <sup>a</sup>	- <sup>b</sup>	-	+
PV-1 <sup>c</sup>	+	-	-	-
FCV	+	-	-	+

<sup>a</sup>Viral capsid function was retained

<sup>b</sup>Viral capsid function was lost

<sup>c</sup>Viral RNA was found to have retained its infectivity

**Table 4.** Summary of disruption of viral capsid and RNA at 37°C inactivation

Virus	Viral capsid integrity			Antigen reaction with antibody
	RNase protection		Attachment to cell receptors	
	Without protease	With protease		
HAV	+ <sup>a</sup>	+	- <sup>b</sup>	+
PV-1	+	+	-	-
FCV	+	+	-	+

<sup>a</sup>Viral capsid function was retained

<sup>b</sup>Viral capsid function was lost

HAV remain the same when incubated at 60°C with a neutral pH for 1 hr; a partial loss of infectivity is found after 10-12 hr of incubation of HAV under the same conditions (Provost et al., 1975; Parry and Mortimer, 1984). Fifty percent of HAV disintegrates at pH 7.0 after incubation for 10 min at 61°C, while 50% of PV-2 disintegrates at temperatures of 43°C given the same pH 7.0 and incubation time. In the presence of 1 M magnesium chloride, the disintegration temperatures of

HAV and PV become 81°C and 61°C, respectively (Siegl et al., 1984). At 56°C, FCV lost 7.5 log<sub>10</sub> infectivity within 60 minutes while, at 70°C, a 3 log<sub>10</sub> reduction of FCV infectivity happened within 1 minute (Doultree et al., 1999). Similar to that of PV, the dissociation of HAV, by heat, involves disruption of the viral capsid and the release of viral RNA, resulting in an accumulation of empty capsid structures. The capsid protein of HAV, partially degraded by heat treatment at 67°C for 10 min or 87°C for 1 min,

remains antigenically reactive to human convalescent sera (Siegl et al., 1984).

#### 4.6 Biodegradation inactivation

Microorganisms, which are indigenously found in the environment, have antiviral properties (Magnusson et al., 1967; Herrmann et al., 1974; Ward and Ashley, 1977; Toranzo et al., 1982; Ward, 1982; Ward et al., 1986; Spillmann et al., 1987; Deng and Cliver, 1992, 1995). One study explains the virucidal effect of ammonia, which is generated by microorganisms, against PV-1, PV-2, coxsackieviruses, echovirus and reovirus type 3 in anaerobically digested sludge (Ward and Ashley, 1977). The author comes to this conclusion on the basis that the key substance is a small, volatile, organic compound, which survives autoclaving and is removed by distillation with activated charcoal.

Indigenous microorganisms found in animal or human feces, slurry, sewage, and sludge are associated with the inactivation of picornaviruses (Ward, 1982; Spillmann et al., 1987; Deng and Cliver, 1992, 1995). The characterization of the agents is primarily based on their molecular size, the susceptibility of their antiviral properties to thermal treatment, susceptibility to antibiotics and any inhibition inactivation by anti-proteases (Ward, 1982; Deng and Cliver, 1995). D values (days for a 90% reduction of virus titers) of PV-1 are 18.7 days, 6.8 days, 1.3 days when PV-1 are inoculated in human mixed waste kept at 21°C, 25°C, and 37°C, respectively (Deng and Cliver, 1992); whereas D values of HAV inoculated in mixed (cattle and swine) slurry, are 10 days and 6 days at 30°C and 37°C, respectively (Deng and Cliver, 1995). Thus, temperature is a factor contributing to viral inactivation in manure; HAV appears to be more resistant to antiviral substances than PV-1 (Deng and Cliver, 1992, 1995). The protease-producing bacteria that are responsible for the inactivation of PV-1 and HAV include *Micrococcus luteus*, *Staphylococcus epidermidis*, *Streptococcus sanguis* group, *Bacillus cereus*, *Bacillus brevis*, *Bacillus subtilis*, *Pseudomonas alcaligenes* (Deng and Cliver, 1992, 1995). Anti-HAV effects of fractions prepared by ultracentrifur-

gation and a series of filtrations with a nominal molecular weight below 1,000, is presumably not an enzymatic action (Deng and Cliver, 1995). Antiviral inactivation by bacteria is found in fresh water, estuarine and seawater (Magnusson et al., 1967; Herrmann et al., 1974; Toranzo et al., 1982; Ward et al., 1986). The enzymatic products of gram-positive and gram-negative bacteria inactivate many enteroviruses and coliphages. The loss of infectivity of echovirus is associated with changes in the viral capsid that result in a lower sedimentation coefficient. The proteolytic cleavages attack VP4 more than VP1 and result in viral RNA exposure to RNase digestion (Herrmann et al., 1974; Ward et al., 1986).

#### 4.7 Drying inactivation

The essence of drying inactivation is based on preventing virus transmission by the food handler, who passes the virus via a fecally soiled finger to the food or food-contact surface (Cliver, 1997). The factors involved are, relative humidity (RH), the temperature of drying, fecal material and the contact surfaces (McCaustland et al., 1982; Keswick et al., 1983; Mbithi et al., 1991; Mbithi et al., 1992; Abad et al., 1994; Doultree et al., 1999; Abad et al., 2001; Gulati et al., 2001). HAV has been shown to be more resistant to drying than FCV, rotavirus, astrovirus, PV and adenovirus. HAV in feces survived up to 30 days after being dried and stored at 25°C and 42% relative humidity (McCaustland et al., 1982), similarly dried FCV survived on coverslip glass for up to 28 days at 20°C (Doultree et al., 1999). These dried state viruses, when in high RH and low temperatures, survived better than in low RH (20%) and at room temperature (Abad et al., 1994; Abad et al., 2001). Fecally suspended Sabin PV-1 survived at 80% and 20% RH for up to 12 and 4 hr respectively (Mbithi et al., 1991). At low RH, PV survived better at 4°C than at 20°C (Abad et al., 2001); the time to a one log<sub>10</sub> reduction of FCV titer at 4°C, 20°C, and 37°C was 11.8, 3.5, days and 7.5 hr, respectively (Doultree et al., 1999). Fecal material played a protective role in extending PV survival during drying inactivation (Keswick et al., 1983; Abad et al., 1994; Abad et al., 2001); the time

to a one log<sub>10</sub> reduction of HAV in fecal material versus buffered medium, on a finger pad, was about 5 hr and 10 min, respectively (Mbithi et al., 1992; Abad et al., 2001). A nonporous surface tended to be more protective for PV (Abad et al., 2001) and FCV (Doultree et al., 1999; Gulati et al., 2001) against drying inactivation, more than a porous surface. After PV was dried, Van der Waals interaction between inorganic solid surfaces and virion, induced spontaneous virion disassembly or the physical disruption of PV virion, while its RNA was left essentially intact (Murray and Laband, 1979; Yeager and O'Brien, 1979).

#### 4.8 Formaldehyde inactivation

Formaldehyde inactivation of picornaviruses is usually used in the preparation of vaccines to prevent the possibility of corresponding infections (Andre et al., 1990; Lemon, 1990; Siegl and Purcell, 1991; Lemon, 1994; Twomey et al., 1995). Formaldehyde inactivation of PV has a direct effect on the viral capsid but an unknown effect on the RNA. The capsid proteins do not disintegrate when the virion is boiled in the presence of strong anionic detergent, in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The sedimentation coefficient of formaldehyde-inactivated PV does not change at < pH 7. Furthermore, RNA is not successfully extracted by phenol, suggesting that the capsid proteins cross-link together and/or link to the viral RNA. This prevents further characterization of the RNA in formaldehyde-inactivated viruses. Despite the fact that previous studies report antigenic inefficiency, the antigenicity of formaldehyde-inactivated PV in a recent study, showed a comparable immune response to those of non cross-linking, inactivated PV e.g. neutral red or N-acetyleneimine (AEI) (Twomey et al., 1995). Similar results are also found with formaldehyde-inactivated foot-and-mouth disease virus (FMDV). In essence, HAV inactivated with formaldehyde for 15 days at 37°C does not show any defective physical properties (Gust et al., 1985). The buoyant density and mean virion diameter remain 1.32-1.34 and 27 nm, respectively.

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