Thermal and pH stability of pestiviruses

K. DEPNER, Th. BAUER and B. LIESS *

Summary: Three strains/isolates of hog cholera virus (HCV) and two strains/isolates each of cytopathogenic (cp) and non-cytopathogenic (ncp) biotype of bovine virus diarrhoea virus (BVDV) were each exposed to pH 3, 3.5 and 4 at 4°C, 21°C and 37°C in a number of combinations. Infectivity titration and half-life determinations following correlation and regression analysis showed a significant temperature-dependent shortening of half-lives within the pH range investigated. At pH 3, mean half-lives were more than tenfold lower when HCV was kept at an ambient temperature of 21°C rather than at 4°C. Additionally, in some of the strains/isolates tested, half-lives of HCV kept at 4°C were four to ten times lower when the pH was raised from 3 to 4. BVDV appeared more sensitive at 4°C and pH 3 than HCV, but equally sensitive at 21°C. Differences in temperature or pH stability between cp and ncp biotypes of BVDV could not be statistically verified although, in general, the cp biotypes seemed to be more stable than the ncp strains/isolates.


INTRODUCTION

Hog cholera virus (HCV) is economically one of the most important pathogens in domestic pigs. Pork and other meat products from pigs infected with HCV are likely to contain the virus. Thus they can serve as a source of clinical outbreaks of hog cholera (classical swine fever) or silent spreading in the case of low virulence strains of HCV (14). Furthermore HCV may be transmitted by indirect contacts after remaining for some time in the environment.

Ambient temperature and hydrogen ion concentration (pH) are important parameters to be measured in order to determine the half-life of HCV based on dose-dependent inactivation. Strains of HCV might differ in their stability (1) in the same way as strains and biotypes of bovine virus diarrhoea virus (BVDV), another pestivirus transmissible to pigs (19).

BVDV derives its practical importance from persistently-infected cattle which are immunotolerant to strains of the non-cytopathogenic (ncp) biotype of BVDV after acquisition in utero during the first trimester of gestation (18, 24). Foetuses born persistently infected can develop inter alia severe symptoms of mucosal disease if the ncpBVDV biotype somehow changes to the cytopathogenic (cp) biotype, or if such animals are superinfected with cpBVDV which has an epitopic structure identical to the persistent ncp biotype (2, 4, 22). Knowledge of marker properties other than

cytopathogenicity would help distinguish between the two biotypes; pH stability or thermostability may serve as such markers. Thermostability may be of particular significance due to the fact that pigs, which have a higher body temperature than cattle, can also be infected with BVDV (18).

Basic data on thermal and pH stability of pestiviruses are required when formulating policies in order to prevent virus dissemination. Information presently available is not comparable and is therefore of only limited value (13).

In the present communication, inactivation rates of HCV and BVDV strains/isolates were biostatistically determined and expressed as the half-life of infectivity for each of the pestiviruses tested.

MATERIALS AND METHODS

Virus strains and isolates

The following HCV strains/isolates were used:

- strain Brescia, made available by the National Swine Fever Laboratory, Perugia, Italy (courtesy of Dr D. Rutili)
- strain 331/USA (21), made available by the National Animal Disease Centre, Ames, Iowa, United States of America (USA) (courtesy of Dr D. Luchsinger)
- isolate Osterode/2699/83, derived from a pig showing symptoms of classical swine fever.

The following BVDV strains/isolates were tested:

a) cytopathogenic biotype
   - strain NADL (7)
   - strain A 1138/69 (6)

b) non-cytopathogenic biotype
   - strain New York 1, made available by the Veterinary Virus Research Institute, Cornell University, Ithaca, New York, USA (courtesy of Dr J.H. Gillespie)
   - isolate 0712/80/Han (17).

The HCV strains/isolates were propagated in cultures of pig kidney (PK15) cells and BVDV strains/isolates in secondary and tertiary foetal calf kidney cell cultures. All culture cells were shown to be free of BVDV prior to inoculation. Culture media consisted of Eagle's Minimum Essential Medium supplemented with 10% bovine serum or 5% foetal calf serum for bovine cells and PK15 cells, respectively. Both types of sera had been tested for freedom from detectable pestivirus-specific neutralising antibodies.

Infectivity titration

For infectivity titration of BVDV as well as HCV, a modified peroxidase linked antibody (PLA) assay (9) was used as described elsewhere (10). Clarified supernatants of virus containing cell culture media were diluted tenfold by stages and assayed in
microtitre plates by simultaneous seeding of virus dilutions and respective culture cells using the method described in previous communications (6, 10). For detection of infected culture cells, a polyclonal anti-pestivirus immunoglobulin derived from pigs was applied. Following intranasal inoculation with BVDV strain Osloss/2482, the pigs were challenged eight weeks later with virulent HCV strain Alfort/187 and bled two weeks later (16). The serum was fractionated and the immunoglobulin preparation conjugated to horseradish peroxidase (3, 11, 23). Infectious doses (TCID₅₀) were calculated using the method described by Reed and Muench (25).

**Experimental design**

Viral suspensions were adjusted to the pH values desired using Britton Welford universal buffer (citric acid, monosodiumphosphate, barbital and boric acid, each in 0.02857 mol solution, adjusted to the desired pH by addition of 0.2 N sodium hydroxide). This was accomplished by mixing one part of viral suspension with nine parts (v/v) of buffers adjusted to pH 3, 3.5, 4 and 7, respectively. Viral suspensions of pH 3, 3.5 or 4 kept at 4°C were submitted to infectivity titrations every three days. Viral suspensions of pH 4 or 7 kept at 21°C and 37°C, respectively, were titrated three times daily.

All viruses tested at the pH values and temperatures indicated in Tables I and II were titrated in triplicate, and arithmetic means determined. Experiments were terminated when virus titres had decreased to less than 10¹⁵ TCID₅₀/0.05 ml, or after eight weeks at the latest. For each virus suspension investigated, half lives were determined on the basis of the calculated regression coefficients.

**Table I**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>pH</th>
<th>Brescia 331/USA</th>
<th>Osterode 2699/83</th>
<th>Mean (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>3</td>
<td>25</td>
<td>66</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>156</td>
<td>197</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>299</td>
<td>257</td>
<td>224</td>
</tr>
<tr>
<td>21°C</td>
<td>3</td>
<td>5 (b)</td>
<td>5 (b)</td>
<td>6 (b)</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>5 (b)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>14</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>ND</td>
<td>77 (b)</td>
<td>24</td>
</tr>
<tr>
<td>37°C</td>
<td>4</td>
<td>ND</td>
<td>0.7 (b)</td>
<td>0.7 (b)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>ND</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

(a) clarified culture media after tenfold dilution in Britton Welford buffers of pH indicated and finally containing 0.5% foetal calf serum

(b) probability P > 0.05

ND no data
Table II

*Half-life values calculated for cell culture-propagated cytopathogenic (cp) and non-cytopathogenic (ncp) bovine virus diarrhoea virus strains/isolates exposed to various temperatures and hydrogen ion concentrations (pH) (a)*

<table>
<thead>
<tr>
<th>Temperature</th>
<th>pH</th>
<th>ncp biotypes</th>
<th>cp biotypes</th>
<th>Mean (hours) ncp/cp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>New York 1 0712/80/Han</td>
<td>NADL A 1138/69</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>3</td>
<td>23 (b)</td>
<td>20 (b)</td>
<td>20/23</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>56</td>
<td>61</td>
<td>50/61</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>151</td>
<td>88 (b)</td>
<td>119/128</td>
</tr>
<tr>
<td>21°C</td>
<td>3</td>
<td>2 (b)</td>
<td>3 (b)</td>
<td>5/7</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>7</td>
<td>8</td>
<td>16/26</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>18/25</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>18</td>
<td>ND</td>
<td>0.7/0.7</td>
</tr>
<tr>
<td>37°C</td>
<td>4</td>
<td>0.7</td>
<td>ND</td>
<td>7/6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

(a) clarified culture media after tenfold dilution in Britton Welford buffers of pH indicated and finally containing 1% foetal calf serum
(b) probability P > 0.05
ND no data

RESULTS

The half-life values calculated for each pestivirus strain/isolate tested are summarised in Tables I and II. Mean half-lives of the HCV or BVDV strains/isolates at various temperatures and pH values are shown in each table. BVDV cp and ncp biotypes were grouped together and mean half-lives listed separately (Table II). To simplify reading, means and ranges of half-life values for HCV and BVDV are given in Tables III and IV.

Table III

*Temperature and pH stability of hog cholera virus (Brescia and 331/USA strains, and Osterode/2699/83 isolate)*

<table>
<thead>
<tr>
<th>Temperature</th>
<th>pH 3</th>
<th>Mean half-life (hours)</th>
<th>pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH 3.5</td>
<td>pH 4</td>
</tr>
<tr>
<td>4°C</td>
<td>70</td>
<td>174</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>(25, 118)</td>
<td>(156-197)</td>
<td>(224-299)</td>
</tr>
<tr>
<td>21°C</td>
<td>5</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>(5, 6)</td>
<td>(5-7)</td>
<td>(10-14)</td>
</tr>
<tr>
<td>37°C</td>
<td>ND</td>
<td>ND</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Ranges of half lives (Table I)
ND no data
TABLE IV

Temperature and pH stability of bovine virus diarrhoea virus (NADL, A 1138/69 and New York I strains, and 0712/80/Han isolate)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>pH 3</th>
<th>Mean half-life (hours)</th>
<th>pH 3.5</th>
<th>pH 4</th>
<th>pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>22</td>
<td>56</td>
<td>124</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(17-26)*</td>
<td>(45-61)</td>
<td>(88-151)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21°C</td>
<td>3</td>
<td>7</td>
<td>11</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2-4)</td>
<td>(3-10)</td>
<td>(8-18)</td>
<td>(18-25)</td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>ND</td>
<td>ND</td>
<td>0.7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(6-7)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* ranges of half lives (Table II)
ND no data

For HCV, the values obtained at 4°C showed a wide variation between strains/isolates at pH 3, less so at pH 3.5 and 4 (Table I). The arithmetic means were about four times higher at pH 4 than at pH 3. At pH 4 the mean half-life values at 21°C showed a more than twenty fold decrease compared to 4°C. Comparison of half-lives between 37°C and 21°C at pH 7 clearly indicates faster thermal inactivation at 37°C.

For BVDV, Table II shows similar variations in temperature and pH stability between strains/isolates as those shown in Table I for HCV. However, while at 21°C and pH 4 the half life values are in approximate agreement with those calculated for HCV, the mean half-life of BVDV at the same pH and 4°C was only half as long as that of HCV. A similar tendency becomes apparent in comparing HCV and BVDV mean half-life values at 4°C and pH 3.5.

In order to demonstrate differences between ncp and cp strains/isolates of BVDV, mean half lives are listed separately in Table II. This shows the constantly higher half-life values for the cpBVDV strains, with the exception of ncp and cp strains/isolates at 4°C and pH 4.

DISCUSSION

Loss of infectivity under certain environmental conditions is of great interest for the control of virus infections such as hog cholera. For bovine virus diarrhoea this aspect is of rather less interest. However, verification of differences between strains/isolates or between biotypes (cp/ncp) with regard to stability at low pH and various temperatures might be of importance. For the detection of such differences, the calculation of half lives by statistical means has been adopted in a previous communication (15).

Despite the use of triplicate titrations of each virus preparation at each temperature and pH, a number of the half-life values indicated in Tables I and II are not statistically significant (P > 0.05). However, they were included in the tables since many of the data communicated in the literature on the stability of pestiviruses are even less reliable.
The techniques previously available for the titration of pestiviruses did not provide the accuracy necessary for the measurement of virus survival rates (13).

Fairly reliable data have been communicated on the pH stability of cell culture-propagated HCV strain Alfort/187 and strain 331/USA, and these were based on inactivation kinetics (1). The residual infectivity indicated a remarkable stability for both strains between pH 3.5 and 10.5 in an ice bath for 40 minutes. A statistical approach was apparently not attempted and the results are therefore difficult to compare with those reported in the present communication. It appears, however, that they do not contradict each other, although enthusiasm concerning verification of differences between strains of HCV cannot be shared with previous authors (1). Differences in pH stability as expressed by variation in half lives of the strains/isolates of HCV (Table I) must be interpreted with caution, although the figures calculated for Brescia strain and Osterode/2699/83 isolate, for example, seem to mirror such differences. The same was true for BVDV strains/isolates and especially for differences between cp and ncp biotypes. With regard to the latter, it cannot be explained why the cp biotype strains generally exhibited longer half-lives than the representatives of the ncp biotype. Nevertheless, all the significant ($P < 0.05$) regression data are in support of differences among pestiviruses not only in pH stability but also in thermostensitivity. Further investigations are needed.

Three temperature grades were selected and tested for their influence on the infectivity of the pestivirus strains/isolates at various pH values so that calculation of correlation and regression coefficients was made possible. Temperatures of 4°C, 21°C and 37°C are of practical importance, e.g. for storage of pestivirus suspensions and inactivation at room temperature, as well as during incubation of cell cultures before penetration and after liberation of virus from infected cells. It became clear that at 4°C, half-lives of HCV vary drastically between pH 3 and 4, but less so at 21°C. At the latter temperature, the decrease of viral infectivity appeared to be more dependent on temperature than pH, e.g. mean half lives of 260 hours at 4°C compared to 11 hours at room temperature (Table I). The half-lives at 37°C and pH 4 were even shorter, and clearly showed the detrimental effect of moderate temperature rather than low pH values on the stability of infectivity.

Since similar findings were encountered with HCV and BVDV under the same conditions (temperature and pH values), it appears justified to claim the results as being typical for pestiviruses. However, ranges should be taken into account whenever required, rather than considering firm figures as typical pestivirus half life values (Tables III and IV). It may be speculated that the half life values reported are higher in environments in which the viruses are better protected, e.g. in the presence of higher concentrations of proteins. In the present study, viral suspensions diluted tenfold in buffers adjusted to the desired pH were used. Therefore the protein and carbohydrate contents were low and comparable to the procedures used by other investigators (1, 8, 15). It can be assumed that the resistance of pestiviruses in meat, for example, is even higher than in aqueous solutions with low concentrations of protective organic substances, such as diluted tissue culture fluids. This is also reflected in a monograph in which the persistence of HCV in products of animal origin was reviewed (5). Lacking proper infectivity assays, the reviewed articles related to dose-dependent inactivation with only semi quantitative results, as they recorded "all or nothing" reactions rather than detailed infective virus concentrations.
In experiments comparable to those reported here, loss of infectivity of HCV at pH 3 to 4 was indicated at 21°C by Loan (20) and Kubin (12). For temperature sensitivity, the only data communicated to date are based on reduction of infectivity of HCV between 4°C and 60°C. While no loss of infectivity was recorded after 2-4 days at 4°C, a titre decrease was noticed at 22°C and 37°C within 2-4 days and 72 hours, respectively. Within an acceptable range, the above data are in accordance with those presented in this communication. However, the present study allows a dose-dependent loss of infectivity to be extrapolated quantitatively from the half-lives at certain pH values and temperatures. Thus, it was possible to demonstrate clearly that temperatures higher than 21°C account for the rapid inactivation of pestivirus, rather than exposure of the virus to pH values in the range of 3 to 4 at the same temperature. Half-lives listed in Tables I to IV can be used for the calculation of the minimum time required for temperature- and pH-dependent loss of infectivity in relation to the original amount of virus under stringent conditions. It must be admitted that in natural environments such as meat or meat products, the effective half-lives might be far higher than those obtained from experiments such as those described in this communication.

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STABILITÉ DES PESTIVIRUS EN FONCTION DE LA TEMPÉRATURE ET DU pH.
K. Depner, Th. Bauer et B. Liess.

Résumé : Trois souches du virus de la peste porcine classique (VPPC), deux souches du biotype cytopathogène et deux souches du biotype non cytopathogène du virus de la diarrhée virale bovine (VDVB) ont été exposées à des pH de 3, 3,5 et 4 à des températures de 4 °C, 21 °C et 37 °C, avec différentes combinaisons de ces deux facteurs. Le pouvoir infectieux et la demi-vie ont été déterminés après des analyses de corrélation et de régression. Un raccourcissement significatif de la demi-vie en fonction de la température est apparu dans la fourchette de pH étudiée. A pH 3, la demi-vie moyenne du VPPC était au moins 10 fois plus courte à la température ambiante de 21 °C qu'à 4 °C. De plus, pour certaines des souches étudiées, la demi-vie du VPPC à 4 °C était encore 4 à 10 fois plus courte lorsque le pH passait de 3 à 4. Le VDVB est apparu plus sensible que le VPPC à 4 °C et à pH 3, mais de sensibilité équivalente à 21 °C. Les différences de stabilité en fonction de la température ou du pH entre les biotypes cytopathogènes et non cytopathogènes du VDVB n'ont pas pu être vérifiées statistiquement mais les biotypes cytopathogènes semblaient en général plus stables que les souches non cytopathogènes.

MOTS CLÉS : Demi-vie - Pestivirus - pH dépendance Température-dépendance Virus de la diarrhée virale bovine - Virus de la peste porcine classique.

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TERMOESTABILIDAD Y ESTABILIDAD EN FUNCIÓN DEL pH DE LOS PESTIVIRUS.
K. Depner, Th. Bauer y B. Liess.

Resumen: Tres cepas/aislados del virus de peste porcina clásica (PPC) y dos cepas/aislados de los biotipos citopatógeno (cp) y no citopatógeno (ncp) del virus de la diarrea vírica bovina fueron expuestos a un pH de 3, 3,5 y 4 a 4°C,
21°C y 37°C en diversas combinaciones. La titulación de infecciosidad y las determinaciones de la vida media después de los análisis de correlación y regresión mostraron una reducción significativa de la vida media en función de la temperatura dentro de la gama de valores de pH investigados. Con un pH 3, la vida media fue en promedio más de diez veces inferior cuando el HCV se mantuvo a una temperatura ambiental de 21°C que cuando esta temperatura fue de 4°C. Además, en algunas de las cepas/aislados examinadas, la vida media del virus de la PPC mantenido a 4°C fue entre cuatro y diez veces inferior cuando el pH se aumentó de 3 a 4. El virus de la diarrea vírica bovina se mostró más sensible a 4°C y pH 3 que el de la PPC, pero ambos virus presentaron la misma sensibilidad a 21°C. Sin embargo, las diferencias de estabilidad de temperatura o pH entre los biotipos cp y ncp no se pudo verificar estadísticamente. En general, los biotipos cp parecían más estables que las cepas/aislados ncp.

PALABRAS CLAVE: Dependencia de la temperatura - Dependencia del pH Pestivirus - Vida media - Virus de la diarrea vírica bovina - Virus de la peste porcina clásica.

REFERENCES


