Median infectious dose of human norovirus GII.4 in gnotobiotic pigs is decreased by simvastatin treatment and increased by age

Tammy Bui,1 Jacob Kocher,1 Yanru Li,1 Ke Wen,1 Guohua Li,1 Fangning Liu,1 Xingdong Yang,1 Tanya LeRoith,1 Ming Tan,2 Ming Xia,2 Weiming Zhong,2 Xi Jiang2 and Lijuan Yuan1

1Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Integrated Life Science Building, 1981 Kraft Drive, Blacksburg, VA 24061-0913, USA
2Division of Infectious Diseases, Cincinnati Children’s Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, OH 45229, USA

Human noroviruses (NoVs), a major cause of viral gastroenteritis, are difficult to study due to the lack of a cell-culture and a small-animal model. Pigs share with humans the types A and H histo-blood group antigens on the intestinal epithelium and have been suggested as a potential model for studies of NoV pathogenesis, immunity and vaccines. In this study, the effects of age and a cholesterol-lowering drug, simvastatin, on the susceptibility of pigs to NoV infection were evaluated. The median infectious dose (ID50) of a genogroup II, genotype 4 (GII.4) 2006b variant was determined. The ID50 in neonatal (4–5 days of age) pigs was \( \approx 2.74 \times 10^3 \) viral RNA copies. In older pigs (33–34 days of age), the ID50 was \( 6.43 \times 10^4 \) but decreased to \( 2.74 \times 10^3 \) in simvastatin-fed older pigs. Evidence of NoV infection was obtained by increased virus load in the intestinal contents, cytopathological changes in the small intestine, including irregular microvilli, necrosis and apoptosis, and detection of viral antigen in the tip of villi in duodenum. This GII.4 variant was isolated in 2008 from a patient from whom a large volume of stool was collected. GII.4 NoVs are continuously subjected to selective pressure by human immunity, and antigenically different GII.4 NoV variants emerge every 1–2 years. The determination of the ID50 of this challenge virus is valuable for evaluation of protection against different GII.4 variants conferred by NoV vaccines in concurrence with other GII.4 variants in the gnotobiotic pig model.

INTRODUCTION

Members of the genus Norovirus in the family Caliciviridae are responsible for the majority of non-bacterial gastroenteritis outbreaks worldwide, with genogroup II, genotype 4 (GII.4) noroviruses (NoVs) being the predominant circulating strain (Koo et al., 2010; Tan & Jiang, 2011). Due to the highly infectious nature and environmental stability of NoVs, they are a serious concern in outbreaks under close-quarter conditions, such as schools, hospitals, cruise ships and retirement communities. Although a number of candidate vaccines have been proposed (El-Kamary et al., 2010; Guo et al., 2008; LoBue et al., 2006, 2009; Tan et al., 2008; Velasquez et al., 2011; Xia et al., 2007), no single NoV vaccine is currently commercially available. Vaccine development has been hampered by the absence of an efficient cell-culture and small-animal model.

In addition to human NoVs, which include GI, GII and GIV, animal NoVs also exist and include those known to infect pigs (GII) (Sugieda et al., 1998), mice (GV) (Karst et al., 2003) and cattle (GIII) (Günther et al., 1984; Woode & Bridger, 1978). Models utilizing these NoV-susceptible animals, along with a few species of non-human primates, have been described to study NoVs and vaccines (Bok et al., 2011; Cheetham et al., 2006, 2007; Jung et al., 2012; Otto et al., 2011; Rockx et al., 2005a; Souza et al., 2007a, b, 2008; Subekti et al., 2002; Takanashi et al., 2011; Ward et al., 2006; Wobus et al., 2006). Ideally, an animal model for human NoVs would exhibit comparable clinical signs and immune responses following NoV infection. Of these animal models, the gnotobiotic (Gn) pig model shares significant
similarities with humans in physiology, immunology, histo-
blood group antigen (HBGA) phenotypes and virus binding
patterns (Cheetham et al., 2007), providing an excellent
animal model for studying human NoVs.

In this study, effects of age and a cholesterol-reducing drug,
simvastatin, on the susceptibility of NoV infection were
evaluated. Our goal was to establish a reliable Gn pig model
of human NoV infection and disease by determining the
median infectious dose (ID$_{50}$) for a recently circulating GII.4
variant (Yang et al., 2010) in neonatal and older Gn pigs
(4–5 and 33–34 days of age, respectively). The use of
simvastatin and other cholesterol-lowering drugs has been
correlated with an increase in susceptibility to NoV infection
(Chang, 2009; Jung et al., 2012). We determined the ID$_{50}$ of
the GII.4 2006b variant (092895) in simvastatin-treated
versus simvastatin-free pigs. One of the applications of the
Gn pig model is to evaluate NoV vaccine efficacy; therefore
ID$_{50}$ at the time of challenge (33–34 days of age for Gn pigs)
is an important parameter to determine. To our knowledge,
this is the first study to determine the ID$_{50}$ of a human NoV
strain in a Gn pig model with or without simvastatin
treatment.

RESULTS

Simvastatin feeding lowers the serum cholesterol
levels

The cholesterol levels among a set of ten pigs tested in one
trial were significantly lowered by simvastatin treatment
at a dose of 8 mg day$^{-1}$ for 11 days (Table 1). The mean
serum cholesterol concentration was reduced from 98 to
79 mg dl$^{-1}$, a mean reduction of 17 %. In another two trials
with a set of six and seven pigs each, the same dosage of
simvastatin treatment reduced the mean serum cholesterol


Table 1. Cholesterol levels (mg dl$^{-1}$) after 11 days of simvastatin feeding (8 mg day$^{-1}$)

<table>
<thead>
<tr>
<th>Pig</th>
<th>Cholesterol levels (mg dl$^{-1}$)</th>
<th>Decrease (%)*</th>
<th>Infection status</th>
<th>Inoculum dose (viral RNA copies)</th>
<th>Peak virus titres shed (RNA copies ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prior to simvastatin</td>
<td>Post-simvastatin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>80</td>
<td>81</td>
<td>−1</td>
<td>No</td>
<td>2.74 × 10$^7$</td>
</tr>
<tr>
<td>2</td>
<td>88</td>
<td>67</td>
<td>24</td>
<td>Yes</td>
<td>2.74 × 10$^4$</td>
</tr>
<tr>
<td>3</td>
<td>99</td>
<td>83</td>
<td>16</td>
<td>Yes</td>
<td>2.74 × 10$^4$</td>
</tr>
<tr>
<td>4</td>
<td>79</td>
<td>74</td>
<td>6</td>
<td>Yes</td>
<td>2.74 × 10$^4$</td>
</tr>
<tr>
<td>5</td>
<td>79</td>
<td>84</td>
<td>−6</td>
<td>Yes</td>
<td>2.74 × 10$^4$</td>
</tr>
<tr>
<td>6</td>
<td>94</td>
<td>89</td>
<td>5</td>
<td>Yes</td>
<td>2.74 × 10$^4$</td>
</tr>
<tr>
<td>7</td>
<td>112</td>
<td>92</td>
<td>18</td>
<td>Yes</td>
<td>2.74 × 10$^4$</td>
</tr>
<tr>
<td>8</td>
<td>117</td>
<td>76</td>
<td>35</td>
<td>Yes</td>
<td>2.74 × 10$^4$</td>
</tr>
<tr>
<td>9</td>
<td>101</td>
<td>78</td>
<td>23</td>
<td>Yes</td>
<td>2.74 × 10$^4$</td>
</tr>
<tr>
<td>10</td>
<td>135</td>
<td>69</td>
<td>49</td>
<td>Yes</td>
<td>2.74 × 10$^4$</td>
</tr>
<tr>
<td>Mean</td>
<td>98</td>
<td>79</td>
<td>17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*A paired Student’s t-test was used to analyse changes in serum cholesterol levels following simvastatin feeding ($P=0.008$).

from 142 to 80 mg dl$^{-1}$ and from 157 to 73 mg dl$^{-1}$, a mean
reduction of 44 and 52 %, respectively (data not shown).

Observation of diarrhoea in both age groups of
Gn pigs following inoculation with NoV

Neonatal pigs and both simvastatin- and non-simvastatin-
fed older pigs exhibited mild diarrhoea (mostly scores of 2)
after NoV inoculation. In the neonatal pigs, 67 % of those
receiving 2.74 × 10$^3$ RNA copies of NoV showed signs of
diarrhoea (Table 2). The incidence of diarrhoea increased
to 100 % in neonatal pigs receiving 2.74 × 10$^5$ RNA copies.
Diarrhoea was also present in older pigs, with or without
simvastatin feeding (Table 3). We observed a tendency for
the older pigs to have looser faeces and consequently
altered the diarrhoea scoring system for these pigs to be
more stringent than the scoring system utilized for the
neonatal pigs. Among the infected older pigs in all dosage
groups, there was a higher occurrence of diarrhoea in the
simvastatin-fed pigs (79 %) compared with the non-
simvastatin-fed pigs (38 %).

Not all of the pigs that shed virus developed signs of
diarrhoea in this study. This most probably reflects an
asymptomatic infection that has likewise been reported in
humans (Gallimore et al., 2004; Lindesmith et al., 2005)
and other animal models (Bok et al., 2011; Rockx et al.,
2005a; Souza et al., 2008).

Gn pigs shed virus in faeces as determined by
reverse transcription-PCR (RT-PCR) and
quantified by TaqMan real-time RT-PCR

Following inoculation, Gn pigs were confirmed to be
infected with NoV by conventional RT-PCR and TaqMan
real-time RT-PCR. Fig. 1 depicts representative large
intestinal content (LIC) samples that were positive for
NoV shedding following inoculation with the 092895 variant. In both age groups without simvastatin treatment, pigs inoculated with a higher dose of virus inoculum displayed a higher, but not significant, rate of virus shedding (Tables 2 and 3a). This was often accompanied with longer duration of shedding, expressed as mean percentage of days with shedding from day 0 post-inoculation (p.i.) until the last rectal swab sample was collected (day 3, 4 or 7 p.i.). Mean percentage of days of virus shedding and diarrhoea are used in Tables 2 and 3 to depict the duration of virus shedding or diarrhoea because the mean number of days of virus shedding or diarrhoea from day 0 to 7 p.i. could not be calculated since a subset of pigs within the different dosage groups was euthanized on days 3–4 p.i. for the pathogenesis study.

Pigs inoculated at 4–5 days of age with $2.74 \times 10^5$ RNA copies were all kept until day 7 p.i. They all shed virus for 3–4 days (42% of the swab days) (Table 2). Only three of the six pigs inoculated with $2.74 \times 10^5$ RNA copies shed virus (all for only 1 day; 11% of the swab days). The $2.74 \times 10^5$ dosage group had significantly higher mean percentage of days with shedding and mean peak titres shed than the $2.74 \times 10^3$ group. Similarly, increased rates and duration of faecal virus shedding were observed in pigs inoculated at 33–34 days of age with $2.74 \times 10^3$ to $2.74 \times 10^6$ RNA copies without simvastatin feeding (Table 3a). The $2.74 \times 10^6$ group had a significantly earlier onset (day 1 p.i.) and longer duration of shedding (mean percentage of days with shedding from day 0 p.i. 75%) than the three lower dosage groups. The virus titres shed in the $2.74 \times 10^6$ group were also significantly higher compared with the two lowest dosage groups. Among the simvastatin-fed pigs, all dosage groups greater than $2.74 \times 10^3$ RNA copies exhibited a 100% shedding rate (Table 3b). The $2.74 \times 10^6$ and $2.74 \times 10^5$ groups had significantly longer duration of shedding (70 and 50% of swab days, respectively) than either of the two lowest dosage groups (13 and 25%, respectively). Significantly longer virus shedding was accompanied with significantly earlier mean onset of shedding (days 1 and 1.3 p.i.) in the two highest dosage groups compared with the two lowest dosage groups (days 3.3 and 3.8 p.i.).

Virus amplification in Gn pigs

It is important to note that the volume of intestinal contents needs to be considered when evaluating the virus load in the pigs. The mean volume of intestinal contents in pigs at 33–34 days of age is approximately 100 ml (Armstrong & Cline, 1977). Based on this estimate, 18 out of 22 NoV-infected (eight non-simvastatin- and 14 simvastatin-fed) pigs, shed a total amount of virus greater than the amount used for inoculation (Table 4). This confirmed that virus replication indeed occurred.

Simvastatin increases susceptibility to NoV infection and the incidence of diarrhoea in Gn pigs

Comparing virus shedding in simvastatin- versus non-simvastatin-fed pigs at each dosage group, the mean percentages of shedding were higher, the mean onset was earlier, the mean percentages of days with shedding were higher, and the peak virus titres were higher in the simvastatin-fed pigs, except for the $2.74 \times 10^6$ dosage group.
Table 3. Summary of NoV shedding in Gn pigs challenged with GII.4 2006b variant (inoculum 092895) at 33–34 days of age with and without simvastatin feeding

Gn pigs were challenged with 2.74 × 10^3 to 2.74 × 10^6 viral RNA copies of NoV 092895 at 33–34 days of age. Rectal swabs were collected daily after challenge to determine virus shedding by conventional and real-time RT-PCR. Virus shedding was also detected in intestinal contents. NA, Not applicable.

(a) Non-simvastatin-fed pigs

| Inoculum dose (RNA copies) | n  | Shedding (%) | Mean onset (days)†† | Mean days with shedding (%)‡‡ | Geometric mean peak titre shed (RNA copies ml^-1)§§ | No. of pigs infected | Infected pigs with diarrhoea ||
|---------------------------|----|--------------|---------------------|-----------------------------|---------------------------------------------------|--------------------|-----------------------------||
| 2.74 × 10^3               | 4  | 25 (1/4) ab  | 6.8 (1.3) a         | 6.3 b                       | 1.29 × 10^4 bc                                    | 1                  | 1                           ||
| 2.74 × 10^4               | 5  | 40 (2/5) ab  | 5.8 (1.4) a         | 9.0 b                       | 5.47 × 10^4 bc                                    | 2                  | 0                           ||
| 2.74 × 10^5               | 3  | 67 (2/3) a   | 5.7 (1.2) a         | 11 b                        | 3.31 × 10^2 ab                                    | 2                  | 0                           ||
| 2.74 × 10^6               | 3  | 100 (3/3) a  | 1.0 (0) b           | 75 a                        | 4.31 × 10^2 a                                    | 3                  | 2                           ||
| Control                   | 11 | 0 (0/11) b   | 8.0 (0) a           | 0 b                         | 0 c                                               | NA                 | NA                          ||
| Total of virus-inoculated pigs | 15 |              |                     |                             |                                                   |                    |                             |

(b) Simvastatin-fed pigs

| Inoculum dose (RNA copies) | n  | Shedding (%) | Mean onset (days)†† | Mean days with shedding (%)‡‡ | Geometric mean peak titre shed (RNA copies ml^-1)§§ | No. of pigs infected | Infected pigs with diarrhoea ||
|---------------------------|----|--------------|---------------------|-----------------------------|---------------------------------------------------|--------------------|-----------------------------||
| 2.74 × 10^3               | 4  | 75 (3/4) a   | 3.8 (1.4) b         | 25 b                        | 1.87 × 10^3 a                                     | 3                  | 2                           ||
| 2.74 × 10^4               | 3  | 100 (3/3) a  | 3.3 (1.5) b         | 13 bc                       | 1.50 × 10^4 a                                     | 3                  | 3                           ||
| 2.74 × 10^5               | 3  | 100 (3/3) a  | 1.3 (0.3) a         | 50 a                        | 9.37 × 10^2 a                                     | 3                  | 2                           ||
| 2.74 × 10^6               | 5  | 100 (5/5) a  | 1.0 (0) c           | 70 a                        | 1.89 × 10^2 a                                     | 5                  | 4                           ||
| Control                   | 11 | 0 (0/11) b   | 8.0 (0) a           | 0 c                         | 0 b                                               | NA                 | NA                          ||
| Total of virus-inoculated pigs | 15 |              |                     |                             |                                                   |                    |                             |

* Proportions in the same column followed by different letters (a, b, c) differ significantly (Fisher’s exact test, P<0.05); while shared letters indicate no significant difference.
† In the groups where some but not all pigs had shedding, the onset for non-shedding pigs was designated 8 for calculating the mean days to onset (pigs were all euthanized before day 7 p.i.). SEM is indicated in parentheses.
‡ Means in the same column followed by different letters (a, b, c) differ significantly (one-way ANOVA, P<0.05); while shared letters indicate no significant difference.
§ Calculated as the percentage of days with shedding from day 0 p.i. until last sample was collected (day 3, 4 or 7 p.i.).
|| Diarrhoea scoring system: 0, solid; 1, pasty; 2, semi-liquid; 3, liquid. Scores of 2 or greater were considered to be diarrhoea.
¶ Pigs were fed 8 mg simvastatin day^-1 for 11 days prior to challenge.
# For this value there was a significant difference when the simvastatin-fed pigs were compared with non-simvastatin-fed pigs.

(Table 3). These differences were not statistically significant except for the mean onset in the 2.74 × 10^5 dosage group, in which the simvastatin-fed pigs had significantly earlier onset of virus shedding than the simvastatin-free pigs (1.3 versus 5.7 days). The total incidence of infection among pigs in the simvastatin-fed group was 93 % compared with 53 % in the simvastatin-free group. The total incidence of diarrhoea among the infected pigs in the simvastatin-fed group was 79 % compared with 38 % in the simvastatin-free group. All these data clearly indicate that simvastatin increased the susceptibility of Gn pigs to NoV infection and the incidence of diarrhoea. However, there were no significant reverse correlations between the peak virus titres shed and the cholesterol levels or the percentage reduction of cholesterol levels in the NoV-inoculated pigs at the time of virus inoculation (Table 1).

**Determination of ID_{50} for the GII.4 092895 variant in neonatal and older pigs, and lowering of the ID_{50} in the older age group by simvastatin**

From the titration of dosages administered to the Gn pigs, the ID_{50} was determined using the Reed–Muench method (Reed & Muench, 1938) (see calculation formula in Methods). Pigs were considered infected if rectal swab or intestinal content samples tested positive by conventional RT-PCR or real-time RT-PCR. The ID_{50} for neonatal pigs was <2.74 × 10^3 viral RNA copies as the lowest dose
administered produced a 50% infection rate. Among the older pigs, simvastatin feeding lowered the ID50 compared with the simvastatin-free pigs. With simvastatin feeding, the ID50 for 33–34 day old pigs was <2.74 \times 10^3 RNA copies as 75% of the pigs were infected at this dose (Table 3). Simvastatin-free pigs at 33–34 days of age had a higher ID50 of 6.43 \times 10^4 RNA copies.

Detection of NoV antigen in the duodenal villi of infected pigs

Presence of human NoV viral capsid protein in the duodenal villi of a pig receiving simvastatin and inoculated with 2.74 \times 10^6 viral RNA copies and euthanized on day 3 p.i. (37 days of age) was demonstrated in Fig. 2. Prominent NoV antigen was detected at the damaged tips of the duodenal villi. NoV antigen was not detected in the duodenum of the mock-inoculated Gn pigs (Fig. 2a) or the jejunum of any pigs (data not shown). Other strains of human NoV have been indicated previously to preferentially infect the proximal small intestine in the Gn pig and calf models based on histopathological changes and occurrence of antigen in enterocytes (Cheetham et al., 2006; Souza et al., 2008).

Cytopathological changes in the duodenum and jejunum in infected pigs

Histologically, no major changes were observed in haematoxylin and eosin stained intestinal sections of the infected pigs (data not shown). However, moderate to severe cytopathological changes were noted in both the duodenum and the jejunum of infected neonatal and older pigs compared with mock-inoculated controls. Representative transmission electron microscope (TEM) images of these intestinal changes from pigs, along with mock-inoculated controls, are depicted in Fig. 3. All of the images in Fig. 3 are from simvastatin-fed pigs. Major changes that were most often observed and were indicative of enteric disease included irregularity, blunting and shortening of microvilli, and cytoplasmic vacuolization. These changes were sometimes associated with signs of necrosis and apoptosis of enterocytes. Mock-inoculated control pigs (simvastatin-fed) did not develop these cytopathological changes, suggesting that the cytopathological changes are not due to simvastatin.

DISCUSSION

In this study, we confirmed that Gn pigs are able to be infected by human NoVs and that this susceptibility can be altered by simvastatin treatment (Jung et al., 2012). Furthermore, we evaluated the ID50 of a particular strain of human GII.4 NoV both with and without simvastatin treatment. Determination of the ID50 is critical in using the animal model in vaccine studies to ensure confident measurements of protective efficacy. Gn pigs are one of the few candidates for an animal model of human NoV infection and disease. All pigs used in this study were A+, H1+ or H2+ (Fig. S1, available in JGV Online). The susceptibility of the pigs to the NoV 092895 variant infection did not differ among the A+, H1+ and H2+ types (data not shown). Differences in susceptibility of infection between the neonatal pigs and the older pigs (both free of simvastatin) was observed, probably reflecting the difference in the maturation status of the immune system of the two age groups. The neonatal pigs were more prone to infection, having a lower ID50 of \( \leq 2.74 \times 10^3 \) viral RNA copies. The ID50 was approximately 23-fold higher at 6.43 \times 10^4 viral RNA copies in the older pigs. The higher dose required to infect the older pigs is probably due to the more developed innate immune system of these pigs. NoV has been reported to affect the young and the elderly to a greater degree, causing more severe symptoms and longer duration of illness (Beersma et al., 2009; Harris et al., 2008; Turcios-Ruiz et al., 2008).

In previous reports, Gn pigs infected by a GII.4 or GII.12 NoV strain developed mild diarrhoea (Cheetham et al., 2006, 2007; Souza et al., 2007a; Takanashi et al., 2011). We demonstrated clearly that simvastatin increased susceptibility to infection and incidence of diarrhoea induced by the GII.4 2006b variant in pigs. The ID50 of the simvastatin-fed pigs inoculated at 33–34 days of age was lower than the simvastatin-free older pigs and possibly equal to or lower than that of the neonatal pigs. However, we did not reach the ID50 dose as the lowest inoculum of 2.74 \times 10^3 RNA copies induced a 75% shedding rate. Simvastatin has been reported to be an immunosuppressive agent through downregulation of innate cytokine IFN-α response and MHC class II dependent T-cell activation, which may explain its ability to increase susceptibility to human NoV infection and the incidence of diarrhoea in Gn pigs.
Table 4. Virus amplification in older Gn pigs with and without simvastatin feeding

Gn pigs were challenged with $2.74 \times 10^3$–$2.74 \times 10^6$ viral RNA copies NoV 092895 at 33–34 days of age. Rectal swabs were collected daily after challenge to determine virus shedding by conventional and real-time RT-PCR. Virus shedding was also detected in intestinal contents.

<table>
<thead>
<tr>
<th>Pig</th>
<th>Inoculum dose (viral RNA copies)</th>
<th>Infection status</th>
<th>Peak virus titres shed (RNA copies ml$^{-1}$)</th>
<th>Total amount of virus shed at peak (RNA copies)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$2.74 \times 10^3$</td>
<td>No</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>$2.74 \times 10^3$</td>
<td>No</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>$2.74 \times 10^3$</td>
<td>No</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>$2.74 \times 10^3$</td>
<td>Yes</td>
<td>$2.73 \times 10^4$</td>
<td>$2.73 \times 10^6$</td>
</tr>
<tr>
<td>5</td>
<td>$2.74 \times 10^4$</td>
<td>No</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>$2.74 \times 10^4$</td>
<td>No</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>$2.74 \times 10^4$</td>
<td>Yes</td>
<td>$1.23 \times 10^4$</td>
<td>$1.23 \times 10^6$</td>
</tr>
<tr>
<td>8</td>
<td>$2.74 \times 10^4$</td>
<td>Yes</td>
<td>$3.99 \times 10^4$</td>
<td>$3.99 \times 10^6$</td>
</tr>
<tr>
<td>9</td>
<td>$2.74 \times 10^4$</td>
<td>No</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>$2.74 \times 10^5$</td>
<td>No</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>$2.74 \times 10^5$</td>
<td>Yes</td>
<td>$7.09 \times 10^3$</td>
<td>$7.09 \times 10^5$</td>
</tr>
<tr>
<td>12</td>
<td>$2.74 \times 10^5$</td>
<td>Yes</td>
<td>$5.13 \times 10^3$</td>
<td>$5.13 \times 10^5$</td>
</tr>
<tr>
<td>13</td>
<td>$2.74 \times 10^5$</td>
<td>Yes</td>
<td>$2.20 \times 10^4$</td>
<td>$2.20 \times 10^6$</td>
</tr>
<tr>
<td>14</td>
<td>$2.74 \times 10^5$</td>
<td>Yes</td>
<td>$7.45 \times 10^4$</td>
<td>$7.45 \times 10^6$</td>
</tr>
<tr>
<td>15</td>
<td>$2.74 \times 10^6$</td>
<td>Yes</td>
<td>$4.87 \times 10^4$</td>
<td>$4.87 \times 10^6$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pig</th>
<th>Inoculum dose (viral RNA copies)</th>
<th>Infection status</th>
<th>Peak virus titres shed (RNA copies ml$^{-1}$)</th>
<th>Total amount of virus shed at peak (RNA copies)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$2.74 \times 10^3$</td>
<td>No</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>$2.74 \times 10^3$</td>
<td>Yes</td>
<td>$5.58 \times 10^3$</td>
<td>$5.58 \times 10^5$</td>
</tr>
<tr>
<td>3</td>
<td>$2.74 \times 10^3$</td>
<td>Yes</td>
<td>$5.61 \times 10^3$</td>
<td>$5.61 \times 10^5$</td>
</tr>
<tr>
<td>4</td>
<td>$2.74 \times 10^3$</td>
<td>Yes</td>
<td>$3.87 \times 10^3$</td>
<td>$3.87 \times 10^5$</td>
</tr>
<tr>
<td>5</td>
<td>$2.74 \times 10^3$</td>
<td>Yes</td>
<td>$7.24 \times 10^3$</td>
<td>$7.24 \times 10^5$</td>
</tr>
<tr>
<td>6</td>
<td>$2.74 \times 10^4$</td>
<td>Yes</td>
<td>$1.72 \times 10^3$</td>
<td>$1.72 \times 10^5$</td>
</tr>
<tr>
<td>7</td>
<td>$2.74 \times 10^4$</td>
<td>Yes</td>
<td>$2.71 \times 10^3$</td>
<td>$2.71 \times 10^5$</td>
</tr>
<tr>
<td>8</td>
<td>$2.74 \times 10^4$</td>
<td>Yes</td>
<td>$1.37 \times 10^3$</td>
<td>$1.37 \times 10^5$</td>
</tr>
<tr>
<td>9</td>
<td>$2.74 \times 10^4$</td>
<td>Yes</td>
<td>$9.15 \times 10^3$</td>
<td>$9.15 \times 10^5$</td>
</tr>
<tr>
<td>10</td>
<td>$2.74 \times 10^5$</td>
<td>Yes</td>
<td>$6.57 \times 10^3$</td>
<td>$6.57 \times 10^5$</td>
</tr>
<tr>
<td>11</td>
<td>$2.74 \times 10^5$</td>
<td>Yes</td>
<td>$3.11 \times 10^3$</td>
<td>$3.11 \times 10^5$</td>
</tr>
<tr>
<td>12</td>
<td>$2.74 \times 10^5$</td>
<td>Yes</td>
<td>$3.13 \times 10^3$</td>
<td>$3.13 \times 10^5$</td>
</tr>
<tr>
<td>13</td>
<td>$2.74 \times 10^5$</td>
<td>Yes</td>
<td>$6.46 \times 10^3$</td>
<td>$6.46 \times 10^5$</td>
</tr>
<tr>
<td>14</td>
<td>$2.74 \times 10^5$</td>
<td>Yes</td>
<td>$1.82 \times 10^3$</td>
<td>$1.82 \times 10^5$</td>
</tr>
<tr>
<td>15</td>
<td>$2.74 \times 10^6$</td>
<td>Yes</td>
<td>$2.10 \times 10^3$</td>
<td>$2.10 \times 10^5$</td>
</tr>
</tbody>
</table>

*Pigs were fed 8 mg simvastatin day$^{-1}$ for 11 days prior to challenge.
†Total amount of virus shed is based on a mean intestinal volume of 100 ml.
diarrhoea up to 5 days post-treatment. It is possible that simvastatin alone might have contributed in part to the increased incidence of diarrhoea in the simvastatin-fed pigs seen in this study. However, signs of diarrhoea were observed in the 092895-variant-infected simvastatin-free pigs, but not in the HS194-infected simvastatin-free pigs. A different GII.4 strain, HS66 (93.9\% capsid protein VP1 sequence identity with 092895), used to infect Gn pigs in previous studies was able to cause mild signs of diarrhoea (Cheetham et al., 2006, 2007; Souza et al., 2007a, b). A GII.12 strain, HS206, has also been reported to cause mild diarrhoea (Takanashi et al., 2011). Therefore, it seems that NoV strains may vary in their ability to cause diarrhoea in Gn pigs.

Strain variability may also affect the levels of virus shedding observed in faeces of NoV-infected Gn pigs. Previous NoV studies using the Gn pig model also reported low genomic copy numbers of virus shedding in faeces (Cheetham et al., 2006, 2007; Souza et al., 2007a). Inoculation of Gn pigs with approximately $2.7 \times 10^6$ genomic equivalents of NoV GII.4 variant HS66 led to 73\% of pigs shedding virus in faeces as detected by RT-PCR (Cheetham et al., 2006). Comparatively, in the older age group in the present study, 100\% of pigs receiving $2.74 \times 10^4$ RNA copies of 092895 with or without simvastatin exhibited faecal virus shedding. With simvastatin feeding prior to virus inoculation, a 100\% shedding rate was seen in pigs receiving as few as $2.74 \times 10^4$ RNA copies. Even with the simvastatin feeding, the possibility does exist that the strain used in this study was better adapted to a porcine host than GII.4 variants used in previous studies and is more infectious in pigs. Yet the virus shedding titres of 092895 in Gn pigs were much lower compared with that of humans, even after higher doses up to $10^6$ viral copies were administered. In human volunteers inoculated with Norwalk virus, the median peak of virus shedding was $9.5 \times 10^{10}$ genomic copies (g faeces)$^{-1}$ (Atmar et al., 2008), whereas the peak titres of virus shed in Gn pigs ranged from $10^3$ to $10^7$ genomic copies (ml faeces)$^{-1}$ in the present study. An emerging GII.12 NoV strain induced shedding titres of $1 \times 10^5–2 \times 10^7$ genomic equivalents ml$^{-1}$ following challenge with $6 \times 10^{10}$ genomic equivalents (Takanashi et al., 2011). Further adaptation may occur with serial passage of the intestinal contents and a subsequent increase in virus replication and shedding occurring in Gn pigs, although it may take many passages for this to progress. Increased susceptibility to human NoV infection was accomplished with administration of simvastatin prior to virus inoculation of the Gn pigs. The geometric mean peak titre shed in the simvastatin-treated animals did not vary greatly among the various infectious doses administered, which may reflect a species barrier in infectivity of NoV. Continued use of simvastatin in conjunction with serial passaging of the intestinal contents in Gn pigs may improve the chance of selecting for a better porcine-adapted strain of 092895.

Presence of abundant NoV antigen after inoculation of Gn pigs with NoV strain 092895 was demonstrated by pigs (Jung et al., 2012; Mach, 2002). Overall though, the ID$_{50}$ of the human GII.4 2006b variant in Gn pigs is higher than that which has been reported in humans since the human NoV has to overcome species barriers in order to infect pigs. For adult humans, Norwalk virus (GI.1) has a low ID$_{50}$ of only 4.8 RT-PCR units (Atmar et al., 2008, 2011).

It is important to note that higher incidences of diarrhoea were observed among the infected pigs treated with simvastatin compared with those not receiving simvastatin. In the study using GII.4 strain HS194 (99.6\% capsid protein VP1 sequence identity with 092895) (Jung et al., 2012), it was observed that simvastatin alone caused mild diarrhoea among the infected pigs treated with simvastatin compared with those not receiving simvastatin.
immunofluorescence staining in the atrophied villi of the duodenum at day 3 p.i. Previous studies in Gn pigs also reported detection of NoV antigen in enterocytes of duodenum and jejunum following infection with either GII.4 strains HS66 or HS194 (Cheetham et al., 2006; Jung et al., 2012), whereas studies of biopsies from

Fig. 3. Cytopathological changes in the duodenum and jejunum of Gn pigs infected with NoV 092895. (a, b) Control pigs that received diluent #5. (c–f) Infected pigs that received 2.74×10^6 RNA copies NoV 092895. All the pigs were treated with simvastatin. Major changes observed in NoV-infected pigs included irregular microvilli (asterisk), cytoplasmic vacuolization (block arrows). Signs of apoptosis (white arrow) included shrunken and darker cells, condensed nuclei and chromatin margination. Signs of necrosis (black arrow) included swollen cells, disrupted cell membranes, loss of organelles and damaged mitochondria. Scale bar, 2 μm.
NoV-inoculated chimpanzees reported that NoV antigens were detected in scattered dendritic cells and B-cells of the lamina propria (Bok et al., 2011). The reason for the discrepancy is unknown. It has been shown that Norwalk virus does not replicate in human macrophages or dendritic cells derived from the peripheral blood of susceptible humans (Lay et al., 2010). One of the possibilities is that NoV replication was not occurring in dendritic cells and B-cells but, instead, these cells took up viral capsid antigen by phagocytosis. In addition, there were differences in NoV genogroup and species in the pig model versus non-human primate model studies. Chimpanzee studies used Norwalk virus (GI.1) and Gn pig studies used GII.4 variants.

Supporting the occurrence of infection, the immunofluorescence and TEM images of the small intestinal tissues from 092895-infected pigs displayed severe cytopathological changes that are characteristic of gastroenteritis. In contrast, HS66-infected pigs showed only very subtle changes in intracellular morphology observable only by TEM (Cheetham et al., 2006). HS194-infected pigs also did not demonstrate histological changes in either the small or large intestine (Jung et al., 2012). These differences in cellular damage may be due to the better adaptability of strain 092895 in porcine hosts compared with HS66 and HS194, or possibly due to the use of simvastatin to increase susceptibility to NoV infection. Most probably, a combination of the two factors contributed to the severity of the cytopathological changes that were observed. In chimpanzees, although virus antigen was detected in the lamina propria, no histopathological changes were observed (Bok et al., 2011). In human volunteers inoculated with Norwalk (GI.1) or Hawaii virus (GII.1), histological changes were evident in proximal intestinal biopsy samples (Karst, 2010). The researchers described blunted villi with decreased length of microvilli, mitochondrial enlargement and paleness, cytoplasmic vacuolization and intracellular oedema. These changes are very similar to what we observed in the duodenum and jejunum of 092895-infected pigs. Thus, the Gn pig–092895-variant challenge model more closely mimics the pathological changes in humans than the HS66–or HS194–Gn pig model and the Norwalk virus–chimpanzee model.

In conclusion, Gn pigs are a better animal model of human NoV infection and disease than other currently available candidates. Using the GII.4 variant 092895, we report similar findings to previous studies of human NoV pathogenesis in Gn pigs, including faecal virus shedding and mild diarrhoea (Cheetham et al., 2006). We demonstrated that simvastatin increased susceptibility to infection and more importantly the incidence of diarrhoea in Gn pigs inoculated with a GII.4 NoV variant. The presence of clinical signs and cytopathological changes following NoV infection is an advantage of this model compared with other animal models. Infection of mice with murine NoVs or non-human primates with human NoVs does not induce gastrointestinal disease (Bok et al., 2011; Rocks et al., 2005a; Ward et al., 2006). In addition, Gn pigs were inoculated orally whereas chimpanzees were inoculated intravenously, which does not mimic the natural route of NoV infection. Using this Gn pig challenge model, NoV vaccine-induced protection against infection and diarrhoea can be evaluated, as well as the immune correlates of protective immunity.

METHODS

**Viruses.** A single pool of human stool containing the GII.4 2006b variant (092895) (GenBank accession no. KC990829) was collected in Dr Xi Jiang’s laboratory in Cincinnati Children’s Hospital Medical Center from a child with NoV gastroenteritis in 2008 and used to orally inoculate Gn pigs. The stool sample was diluted to 10% in diluent #5 (minimal essential medium with 1% penicillin-streptomycin and 1% HEPEs) and treated by high-speed centrifugation to remove bacteria. Sterility was confirmed by culturing the treated inoculum on blood-agar plates and in thioglycollate medium. Absence of other contaminating viruses was confirmed by testing on a Virochip Microarray (University of California, San Francisco Viral Diagnostics and Discovery Center). Endotoxin levels were measured by a limulus amoebocyte lysate test (ToxinSensor chromogenic LAL endotoxin assay kit; GenScript). The inoculum contains an endotoxin level of 1 EU ml⁻¹, which is below the recommended level for live attenuated vaccines (<200 EU ml⁻¹) (Brito & Singh, 2011).

**Inoculation of Gn pigs.** Near-term Large White cross pigs were derived by hysterectomy and maintained in germ-free isolator units as described previously (Meyer et al., 1964). A⁺/H⁻ pigs were orally inoculated at either 4–5 or 33–34 days of age with 2.74 × 10⁶–2.74 × 10⁷ viral RNA copies of GII.4 variant 092895 suspended in 5 ml medium (diluent #5). Control pigs received diluent #5 only. To neutralize stomach acids, 10 min prior to inoculation 4 ml 200 mM sodium bicarbonate was given. Pigs were euthanized at day 3, 4 or 7 p.i. to collect intestinal contents and tissues. All animal experimental procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committees of Virginia Tech.

**Simvastatin.** To evaluate the effect of simvastatin on increasing virus infectivity (Chang, 2009; Jung et al., 2012), a subset of pigs receiving 2.74 × 10⁶–2.74 × 10⁷ viral RNA copies of GII.4 variant 092895 were treated with simvastatin (Dr Reddy’s Laboratories), which was given orally at a dose of 8 mg day⁻¹ for 11 days prior to virus inoculation with NoV. As the younger pigs were inoculated at 4–5 days of age, they did not receive any simvastatin treatment. Briefly, 80 mg tablets of simvastatin were dissolved in 100% ethanol to a concentration of 8 mg ml⁻¹, filtered for sterility purposes and diluted in 6 ml diluent #5 prior to feeding. Serum cholesterol levels of subsets of pigs were measured by the Virginia-Maryland Regional College of Veterinary Medicine Veterinary Hospital laboratory pre- and post-treatment with simvastatin to confirm reduction of cholesterol levels.

**Blood typing of Gn pigs by PCR and immunofluorescence assay.** Prior to inoculation, Gn pigs were confirmed to be A⁺ or H⁺ in blood type by PCR and an immunofluorescence assay (Fig. S1). It has been shown that HBGA phenotype influences susceptibility to NoV infection and that A⁺ or H⁺ pigs are more likely to be infected than A⁻ or H⁻ pigs (Cheetham et al., 2007; Huang et al., 2003, 2005; Hutson et al., 2002; Marianneau et al., 2002; Rocks et al., 2005b; Tan & Jiang, 2005; Tian et al., 2007).

PCR blood typing was conducted with DNA isolated from whole blood using DNAzol Genomic DNA Isolation Reagent (Molecular Research
Center) and following the manufacturer’s instructions for micro-isolation of DNA from whole blood. At least 50 ng genomic DNA was required to perform the PCR. Forward primer ABO4s (5’-AGCTTGGTCTGGAGACGGCAGA-3’) and reverse primer ABO5a (5’-CAGTGTCCTCCTCATGCCCACAC-3’), designed by Revivior and producing a 500 bp product, were used to determine whether the pigs were A+ or A−. Internal control primers Pig5’ (5’-CCCTGGAACTCTGGACCTGTC-3’) and Pig3’ (5’-CTGACGTCAGACCAAGGGTCT-3’), producing a 300 bp product, were also included. PCR conditions were as follows: initial denaturation at 94°C for 3 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min, and a final extension of 72°C for 10 min. To determine the H phenotype of Gn pigs and confirm the PCR results, buccal cells were collected and immunofluorescently stained as described previously (Busch et al., 2006). Briefly, the cheeks of Gn pigs were swabbed to collect buccal cells. Swabs were then swirled in 1 ml PBS, centrifuged to pellet the cells, subsequently washed twice with PBS to remove any traces of milk and resuspended in 20 μl PBS. Slides were prepared using 2 μl drops of the buccal cell suspension. A, H1 and H2 HBGA phenotypes were detected with mouse anti-human A (1:50 dilution; Covance Research Products), H1 (1:50 dilution; Covance Research Products) and H2 (1:200 dilution; Abcam). Slides were counterstained with propidium iodide (Invitrogen) and observed using a fluorescent microscope.

Assessment of NoV diarrhea. Gn pigs were rectally swabbed daily following inoculation with GII.4 human NoV variant 092895 to assess diarrhea and NoV shedding. The scoring system was adapted from Cheetham et al. (2006) and amended depending on the age of the pigs due to the change in consistency of the faeces with maturity. Pigs inoculated at 4–5 days of age were scored based on the following system: 0, solid; 1, semi-solid; 2, pasty; 3, semi-liquid; 4, liquid. Pigs inoculated at 33–34 days of age were scored based on a more stringent system: 0, solid; 1, pasty; 2, semi-liquid; 3, liquid. For both scoring systems, a score of 2 or greater was considered to be diarrhea. Rectal swabs were also collected weekly for a sterility test on blood-agar plates and in thioglycollate medium.

Detection of NoV shedding by RT-PCR. Rectal swabs were swirled in 1 ml PBS and large and small intestinal contents (LIC and SIC, respectively) were collected as described above and below the 50 % end point:

\[
\text{ID}_{50} = \left( \frac{\log \text{dilution above } 50\%}{-\log \text{dilution below } 50\%} \right) \times (\text{PD} \times \log \text{dilution factor})
\]

Dilution factor is the fold difference between the titres for above 50 % and below 50 % infected.

Detection of NoV antigen in intestinal tissues by indirect immunofluorescence. Duodenum and jejunum samples were collected upon euthanasia and fixed in 4 % paraformaldehyde-PBS, dehydrated in a graded ethanol series and embedded in paraffin blocks. Sections were cut and collected on positively charged slides. The staining procedure was adapted from previous studies (Cheetham et al., 2006; Jung et al., 2012). NoV antigen was detected using guinea pig anti-NoV serum against a mixture of recombinant virus-like particles from nine GI and GII NoVs including VA-387 (GII.4), Grimsby (GII.4), MOH (GII.5), VA-207 (GII.9), (GII.1), Mexico (GII.3), Norwalk (GI.1) and Hawaii VA-115 (GI.3) produced in Dr Xi Jiang’s laboratory at the Cincinnati Children’s Hospital Medical Center] diluted 1:3000.

Evaluation of cytopathological changes by TEM. Duodenum and jejunum samples collected upon euthanasia from both infected and mock-inoculated Gn pigs were fixed in 3 % glutaraldehyde/PBS fixative solution for TEM as described previously (Cheetham et al., 2006).

Statistical analysis. Proportions of virus shedding and diarrhea among treatment groups were compared using Fisher’s exact test. The mean percentage of days of virus shedding and diarrhea along with peak titres shed among the treatment groups were compared using a known NoV-positive human stool sample was used as a positive control.
one-way ANOVA general linear model, followed by Duncan’s multiple range test. A two-tailed paired Student’s t-test was used to analyse changes in serum cholesterol levels following simvastatin feeding. Statistical significance was assessed at $P<0.05$ for all comparisons. All statistical analyses were performed using SAS program 9.3 (SAS Institute).

ACKNOWLEDGEMENTS

We thank Dr Marlice Vonck, Dr Kevin Pelzer, Dr Sherrie Clark, Pete Jobst, Andrea Aman, Kim Allen and Shannon Viets for animal care. We thank Kathy Lowe for assistance in transmission electron microscopy. This work was supported by a start-up fund from Virginia Polytechnic Institute and State University to L.Y. and an NIH grant from NIAID (National Institute of Allergy and Infectious Diseases), NIH (R01AI089634-01) to X.J. and L.Y.

REFERENCES


norovirus vaccines induce strong mucosal and systemic blocking antibodies against multiple strains. *Vaccine* 24, 5220–5234.


