Antiviral activity of chloroquine against human coronavirus OC43 infection in newborn mice

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ABSTRACT

Until recently, human coronaviruses (HCoV), such as HCoV-OC43, were mainly known to cause 15 to 30% of mild upper respiratory tract infections. In recent years, the identification of new HCoVs, including severe acute respiratory syndrome coronavirus, revealed that HCoVs can be highly pathogenic, and can cause more severe upper and lower respiratory tract infections, including bronchiolitis and pneumonia. To date, no specific antiviral drugs are available, neither to prevent nor to treat HCoV infections. We demonstrate that chloroquine, a widely used drug with well known antimalarial effects, inhibits the HCoV-OC43 replication in HRT-18 cells, with a 50% effective concentration of $0.306 \pm 0.0091$ (SD) $\mu$M and a 50% cytotoxic concentration of $419 \pm 192.5$ (SD) $\mu$M, resulting in a selectivity index of 1369. Further, we investigated whether chloroquine could prevent death in newborn mice as a result of infection with HCoV-OC43. Our results show that a lethal HCoV-OC43 infection in newborn C57BL/6 mice can be treated with chloroquine, transplacentally acquired or via the maternal milk. The highest survival rate (98.6%) of the pups was found when mother mice were treated daily with a concentration of 15 mg/kg chloroquine. Survival rates declined in a dose-dependent manner, with 88% survival when treated with 5 mg/kg chloroquine, and 13% survival when treated with 1 mg/kg chloroquine. Our results show that chloroquine can be highly effective against HCoV-OC43 infection in newborn mice and may be considered as a future drug against HCoVs.

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INTRODUCTION

Coronaviruses are large, enveloped single-stranded positive sense RNA viruses with a genome of approximately 30 kb in length, the largest found in any of the RNA viruses. The genus *Coronavirus* belongs to the family *Coronaviridae* in the order *Nidovirales*. The coronaviruses are classified into three groups based on genetic and serological relationships. Group 1 contains the porcine epidemic diarrhea virus (PEDV), porcine transmissible gastroenteritis virus (TGEV), canine coronavirus (CCoV), feline infectious peritonitis virus (FIPV), human coronavirus 229E (HCoV-229E), and human coronavirus NL63 (HCoV-NL63). Group 2 contains the murine hepatitis virus (MHV), bovine coronavirus (BCoV), human coronavirus OC43 (HCoV-OC43), rat sialoadenitis virus (SDAV), porcine hemagglutinating encephalomyelitis virus (PHEV), canine respiratory coronavirus (CRCoV), and equine coronavirus (ECoV). The SARS coronavirus (SARS-CoV) is considered as a distant member of group 2, and is therefore placed in a subgroup, 2b (13). Group 3 thus far only contains avian coronaviruses such as infectious bronchitis virus (IBV) and turkey coronavirus (TCoV). Within group 2, HCoV-OC43 is most closely genetic related to bovine coronavirus (BCoV) (33). A comparative analysis of the complete genomes of HCoV-OC43, BCoV, and PHEV demonstrated a high genetic similarity among these three coronaviruses in more than two-third of their genomes, except in the hemagglutinin-esterase and spike gene region, in which PHEV was remarkably divergent from HCoV-OC43 and BCoV (30,31,33).

Human coronaviruses (HCoV) cause respiratory infections, but gastroenteritis and neurological disorders can also occur (3,20). Until now, five human coronaviruses have been described. HCoV-OC43 and HCoV-229E are responsible for 10 to 30% of all common colds, and infections occur mainly during the winter and early spring (21). In 2003, a novel HCoV, displaying only distant antigenic and genetic similarities with the two previously known HCoVs, were identified as the
causal agent of the severe acute respiratory syndrome (SARS) and causes severe lung disorder, leading in some cases to systemic infection and eventually death in about 10% of cases (19). The two following years after the SARS outbreak, two additional previously unrecognised coronaviruses affecting humans, HCoV-NL63 and HCoV-HKU1, have been identified (29,35). HCoV-NL63 infection is related to acute respiratory dysfunction in infected individuals. Furthermore, HCoV-NL63 was identified as the major pathogen responsible for croup in young children (9,10,22). The clinical features of HCoV-NL63 infections appear to be more severe than those commonly attributed to infections by HCoV-OC43 and 229E (4,12,29). Infection with HCoV-HKU1 is mostly associated with bronchiolitis and pneumonia (35,36).

Although coronaviruses have been recognized as human pathogens for about 50 years, no effective treatment strategy has been approved. This shortcoming became evident during the SARS-CoV outbreak and was the start of numerous studies. Nevertheless, 5 years after the outbreak, we are still lacking an effective, commercially available drug. Chloroquine is a clinically approved drug effective against malaria, and is known to elicit antiviral effects against several viruses, including HIV type 1 (HIV-1) (23,26,28), hepatitis B virus (18), and herpes simplex virus type 1 (27). Savarino and colleagues hypothesized that chloroquine might be of some use for the clinical management of SARS (25). Moreover, chloroquine was reported to inhibit the replication of HCoV-229E (7) and SARS-CoV (16) in vitro. In a previous study, we showed that the EC50 of chloroquine (8.8 ± 1.2 µM) for the inhibition of the SARS coronavirus in vitro was significantly lower than its cytostatic activity (261.3 ± 14.5 µM) yielding a selectivity index of 30, and that this EC50 approximates the plasma concentrations of chloroquine reached during treatment of acute malaria. Addition of chloroquine to infected cultures could be delayed for up to five hours postinfection, without an important drop in antiviral activity ((16). The antiviral effects of chloroquine against HIV-1 replication are currently being tested in
clinical trials (25). Chloroquine is a weak base that increases the pH of acidic vesicles. When added extracellularly, the non-protonated portion of chloroquine enters the cell, where it becomes protonated and concentrated in acidic, low-pH organelles, such as endosomes, Golgi vesicles, and lysosomes. Chloroquine can affect virus infection in many ways, and the antiviral effect depends in part on the extent to which the virus utilizes endosomes for entry. Besides a direct antiviral effect, chloroquine is endowed with an immunomodulatory activity, suppressing the production and release of tumor necrosis factor α and interleukin 6, which mediate the inflammatory complications of several viral diseases (25).

In the present study, we further investigate the anti-coronaviral properties of chloroquine, by testing the in vitro antiviral activity of chloroquine against HCoV-OC43. In addition, we developed a lethal in vivo challenge model to test the antiviral effect of chloroquine against HCoV-OC43.
MATERIALS AND METHODS

Test compounds. We tested chloroquine phosphate (7-chloro-4-[(4-diethylamino)-1-methylbutyl]amino] quinoline phosphate, Alpha pharma, Braine-l’Alleud, Belgium).

Virus and cells. The OC43 strain was originally obtained from the American Type Culture Collection (ATCC, Rockville, MD). HRT-18 cells (ATCC, Rockville, MD, USA) were propagated in minimal essential medium (MEM; Gibco, Life Technologies, Rockville, MD, USA) supplemented with 10% fetal calf serum (FCS; Integro, Zaandam, The Netherlands), 1% L-glutamine (Gibco), and 1.4% sodium bicarbonate (Gibco). Virus-infected cells were maintained in MEM supplemented with 2% FCS. For antiviral activity and cytotoxicity measurements, supernatant was harvested after 4 days of incubation at 37°C in the presence of 5% CO₂.

Real-time RT-PCR. The methodology of the real-time RT-PCR assay has been described previously (32). Briefly, the quantitative RT-PCR was performed in a 25-μl reaction mixture with 5 μl extracted RNA, 12.5 μl of Eurogentec One-Step Reverse Transcriptase qPCR master mix containing ROX as a passive reference dye, 0.125 μl Euroscript + RT & RNase inhibitor (Eurogentec, Seraing, Belgium), 300 nM forward and reverse primers, and 200 nM MGB probe. Amplification and detection were performed in a ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). In order to allow absolute HCoV-OC43 quantitation, cRNA standards were constructed and used for the generation of a standard curve, as described previously (32).

qRT-PCR-based antiviral activity assay. Antiviral measurements were based on the reduction in viral titer of coronavirus infected cells. HRT-18 cells were seeded at a density of 6 x 10⁴ cells per well into a 24-well culture plate. After 4 days of growth, cells were infected with 1 x 10⁵ HCoV-OC43 copies per ml in the presence of various concentrations of chloroquine ranging from 6
0.032 to 500 μM. After 4 days of incubation at 37 °C in the presence of 5% CO₂, cell supernatants were collected. Viral RNA was extracted using the QIAamp viral RNA kit (Qiagen, Venlo, The Netherlands) to determine the viral RNA load in the cell supernatant by using the quantitative RT-PCR described above.

**Cytotoxicity assay.** Cytotoxicity measurements were based on the viability of HRT-18 cells in the presence of various concentrations of the test compounds. Four days after addition of the compounds, the number of viable cells was quantified by a tetrazolium-based colorimetric method, in which the reduction of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) dye (CellTiter 96 AQueous One Solution kit, Promega, The Netherlands) by mitochondrial dehydrogenases to a soluble colored formazan was measured in a spectrophotometer (Multiskan EX, Thermo Labsystems, Belgium) at 492 nm. The 50% cytotoxic concentration (CC50) was defined as the concentration of the compound that reduced cell viability by 50%.

**Time-of-addition assay.** Subconfluent monolayers of HRT-18 cells in 24-well plates were infected with 4 x 10⁷ HCoV-OC43 genome equivalents per ml MEM. After 30 minutes of adsorption, cell monolayers were washed five times with MEM. Chloroquine was added in triplicate at a concentration 30-fold above the EC₅₀, at the time of infection or at different time points thereafter. Twenty-eight hours after infection cell supernatant was collected. Viral RNA was extracted using the QIAamp viral RNA kit (Qiagen), to determine the viral RNA load in the cell supernatant by using the quantitative RT-PCR described above.

**Mouse infection studies.** Six-week-old male and female C57BL/6 mice (Elevage Janvier, Le Genest Saint Isle, France) were coupled. Female pregnant mice were daily injected subcutaneously with 200 μl of different dilutions of chloroquine (corresponding to 0, 1, 5 or 15
mg/kg) starting from 2 days before labour. Subsequently, five day old C57BL/6 pups were
inoculated intracerebrally with 10 µl of virus dilution (i.e. $1 \times 10^3$ HCoV-OC43 viral copies/10 µl)
The suckling mice were daily monitored for mortality. The surviving mice were followed during
60 days after the infection. In the switching experiments, 3 groups of pregnant C57BL/6 mice
were used. One group of mice were treated once with 15 mg/kg chloroquine 1 day pre partum,
one group of mice were treated daily with 15 mg/kg post partum, and one group did not received
any chloroquine treatment and served as infection control. At the day of birth, the litter of group 1
were switched with the litter of group 2 and vice versa. Eventually, the newborns in group 1
received chloroquine only via the maternal milk and the newborns in group 2 received
chloroquine only transplacental. After 5 days, the 5-days-old suckling mice were inoculated
intracerebrally with $1 \times 10^3$ genome copies of HCoV-OC43. All mice were treated according to
the laboratory animal control guidelines of our institute, which conforms to those of the European
Commission. All animal experiments were carried out in a BSL-2 facility.

**Statistical analysis.** All statistical tests were carried out using GraphPad Prism version 4.00. The
significance level was set at $p<0.05$. 
RESULTS

**In vitro antiviral activity of chloroquine.** QRT-PCR provides a useful tool in determining the antiviral activity of a compound against HCoV-OC43. In this antiviral assay, the inhibitory effect of the compound on viral replication is calculated by quantitation of the viral growth (i.e. RNA genome equivalents) in the presence of the compound versus the viral growth without the compound (i.e. the positive control). The 50% effective concentration (EC$_{50}$) of the compound was defined as the concentration that inhibited the viral RNA increase by 50%. To allow absolute HCoV-OC43 RNA quantitation, cRNA standards were constructed and used for the generation of a standard curve. A chloroquine concentration above 0.16 µM results in a decline in the number of HCoV-OC43 copies (data not shown). The EC$_{50}$ of chloroquine is calculated to be 0.306 ± 0.091 (SD) µM. The chloroquine concentration that reduced cell viability to 50% (CC$_{50}$ or 50% cytotoxic concentration) was 419.0 ± 192.5 (SD) µM. Chloroquine inhibits the in vitro replication of HCoV-OC43 with a selectivity index of 1369. The selectivity index is determined as the ratio of the CC$_{50}$ to the EC$_{50}$.

**Antiviral activity of chloroquine administered at different time points after coronavirus infection.** A time-of-addition assay was performed to determine the *in vitro* viral activity of chloroquine when added at various time points after virus infection. For this purpose HRT-18 cells were infected with HCoV-OC43. Chloroquine was added at a concentration of 10 µM (i.e. 32.7 times the EC$_{50}$) at different time points after infection. Viral RNA levels in the cell supernatants were determined 28 h post-infection. The measurement of the viral load shows that chloroquine is required at the moment of infection to block the HCoV-OC43 replication (Fig. 1). When chloroquine was added at the time of infection, viral RNA could still be detected, but the viral load was reduced 100-fold in comparison to the positive control. At later time points, a loss of the antiviral activity of chloroquine was noted.
Antiviral activity of chloroquine against a lethal HCoV-OC43 infection in newborn C57BL/6 mice. We investigated whether chloroquine could prevent death in newborn mice as a result of infection with HCoV-OC43. C57BL/6 mice were selected in view of their susceptibility to HCoV-OC43 (14,15). Pregnant mice were treated subcutaneously 1 day pre or 1 day post partum with chloroquine (0, 1, 5 or 15 mg/kg). Subsequently, 5-day-old suckling mice were inoculated intracerebrally with 1×10^3 genome copies of HCoV-OC43. This setting allows us to distinguish between chloroquine transmitted through the placenta and the maternal milk and chloroquine solely transmitted through the maternal milk. In litters of untreated mothers, all the pups died within 6 days after HCoV-OC43 challenge (Fig. 2). A 100% survival rate was seen in the litters of the mother mice that were treated with 15 mg/kg chloroquine pre partum (Table 1 and Fig 2A) (97.4% survival of litters from post partum treated mothers). Survival rates of the pups declined in a dose dependent manner, with 88.1% survival (92.9% when mother mice were treated pre partum and 83.3% survival when treated post partum) when treated with 5 mg/kg chloroquine, 13.5% survival (33.3% when mother mice were treated pre partum and 0% when treated post partum) when treated with 1 mg/kg chloroquine. A Longrank test indicated that the survival curve of litters that were treated pre partum with 15mg/kg chloroquine was significant different from the survival curves of the pups that were treated pre partum with 5 mg/kg (p=0.0237), 1 mg/kg (p<0.0001) or 0 mg/kg (p<0.0001). The survival curve of the post partum treated litters with 15 mg/kg in comparison to the survival curves of the post partum treated litters with respectively 5 mg/kg (p=0.0053), 1 mg/kg (p<0.0001) or 0 mg/kg (p<0.0001) were also significant different.

Chloroquine transfer via the placenta and via the maternal milk. Since the survival rate was higher in newborn mice from pre partum treated mothers, we investigated the role of transplacentally versus orally (via the maternal milk) acquired chloroquine. In this study, a dose
of 15 mg/kg chloroquine was given to the mother mice, because this dose resulted in nearly 100% survival of the pups. For the setup of this test, pregnant mice were divided into 3 groups. Only the mother mice from group 1 were treated with chloroquine one day pre partum. At the day of birth, the pups from treated mothers (group 1) were switched to untreated mother mice (group 2) and vice versa. Eventually, the newborns in group 1 received chloroquine only via the maternal milk and the newborns in group 2 received chloroquine only transplacental. Subsequently, 5-day-old suckling mice were inoculated intracerebrally with $1 \times 10^3$ genome copies of HCoV-OC43. In litters of untreated mothers (group 3), all the pups died within 6 days after HCoV-OC43 challenge (Fig. 3). The survival rate of newborn mice that received the chloroquine only transplacental (group 2) was 0% (0/27) (Table 1). The survival rate of newborn mice that received the chloroquine via the maternal milk (group 1) was 69.0% (20/29). A Longrank test indicated that there was a significant difference between the survival curve of the pups that received chloroquine transplacental and the survival curve of the pups that received the chloroquine only via the maternal milk ($p<0.0001$).
As HCoVs are no longer considered to be as harmless as previously assumed, and as they can be
associated with severe respiratory tract infections, the identification of compounds effective
against these viruses becomes an important issue. Chloroquine is a clinically approved drug
effective against malaria, and is known to elicit antiviral effects against several viruses, including
the SARS-CoV and HCoV-229E (7,16,17). However, in vivo studies were unable to show the
antiviral effectiveness of chloroquine against the SARS-CoV (6). In the present study, we
investigated the anti-coronaviral properties of chloroquine, by testing its in vitro and in vivo
antiviral activity against the group 2 HCoV-OC43.

With the in vitro antiviral experiments, chloroquine showed in vitro antiviral properties against
HCoV-OC43 replication in HRT-18 cells with an EC50 of 0.306 µM. The 50% cytotoxic
concentration (CC50) was 419 µM, resulting in a selectivity index (SI) of 1369. In comparison to
the anti-SARS-CoV activity of chloroquine, with an EC50 of 8.8 µM and a SI of 30 (16),
chloroquine is a more potent inhibitor of the HCoV-OC43 replication. The exact mechanism of
antiviral intervention of chloroquine is not yet elucidated but is possibly a multi-target
mechanism, depending on the time-point at which the drug is added. When added during and
shortly after infection, chloroquine probably affects the endosome-mediated fusion, and when the
drug is given after this first target, it can still act on later stages of the viral life cycle, as reported
for other viruses (25). For the anti-SARS-CoV activity, chloroquine was proven equally active
when added during virus adsorption or 1 hour after infection. Later on, a gradual loss of the
antiviral activity was seen (16). Vincent and colleagues found that chloroquine is effective in
preventing SARS-CoV infection in cell culture. When the drug is added 24 h prior to the
infection at a concentration of 10 µM, the infectivity was completely eliminated. Moreover, they
found that chloroquine was significantly effective even when the drug was added 3–5 h after
infection, suggesting an antiviral effect even after the establishment of infection. Since they obtained similar results by NH₄Cl treatment of Vero cells, the underlying mechanism(s) of action of chloroquine and NH₄Cl might be similar and might be attributed to the alkaline properties of both compounds (34). Interestingly, our time-of-addition experiments with HCoV-OC43 pointed out that chloroquine was required at the moment of infection to block the HCoV-OC43 replication. When added at the time of infection, chloroquine reduced the viral load with two log in comparison to the positive control. At later time points, a loss of antiviral activity of chloroquine was noted. Thus, chloroquine appears to act only on the entry of HCoV-OC43 and not on the later stages of the replication cycle.

Because of this markedly broad activity spectrum against coronaviruses, we developed an in vivo model to test the antiviral properties of chloroquine against HCoV-OC43. Our results show that a lethal HCoV-OC43 infection in newborn C57BL/6 mice can be treated with an effective antiviral concentration of chloroquine, transplacentally acquired or through the maternal milk. Pregnant mice were treated once with several doses of chloroquine pre or post partum. Since the survival rate was higher in newborn mice when the mother was treated pre partum (Fig. 2A), we investigated the role of the transplacentally versus the orally (i.e. via the maternal milk) acquired chloroquine in a second experiment. In litters of untreated mothers, all the pups died within 6 days after HCoV-OC43 challenge. All newborn mice that received chloroquine exclusively transplacentally died (Fig 3). A possible reason for the 100% mortality can be that chloroquine is not transferred through the placenta. This is highly unlikely, because almost all xenobiotics that are given during pregnancy can enter the fetal blood circulation through passive diffusion. Moreover, transplacental distribution of chloroquine has already been documented in humans and rabbits (1,2,11). In sheep, on the other hand, the transfer rate of chloroquine from the mother to the foetus was low (5). No data are available for transplacental transfer in mice although
placentary transfer of chloroquine in mice seems likely, given the higher survival rate of newborn mice when treated both before and after birth in comparison with newborn mice solely treated after birth.

The 100% mortality of the pups that received the chloroquine only transplacentally is most likely attributable to an ineffectively low concentration of chloroquine reached in the newborn mice. Chloroquine accumulates in tissue and organs of the mother and the foetus and shortly after administration, a balance between mother and foetus will be reached (2,24). The concentration of chloroquine reached after equilibrium in the foetus, in case of treatment with 15 mg/kg chloroquine pre partum, is possibly not sufficient to protect against a lethal HCoV-OC43 infection. A higher dose of chloroquine is in this animal model not an option, since previous experiments with 30 mg/kg chloroquine administered pre or post partum, revealed that this dose was toxic for the mother mice and lethal for the pups (data not shown).

In humans, chloroquine accumulates in the milk glands, resulting in high chloroquine concentrations in the maternal milk (1,8). The chloroquine concentration in the blood of the suckling mice will increase with the amount of maternal milk they drink. In this way, an antiviral effective chloroquine concentration can be reached.

The survival rate of newborn mice that were switched after birth and received chloroquine only via the maternal milk (the mothers were treated with 15mg/kg chloroquine) was 69.0% (20/29). This rate is lower than the survival rate of newborn mice whose mothers received chloroquine post partum (97.4 %). A first reason for this lower survival rate in the switched litters can be attributed to the fact that the mother mice, treated pre partum with chloroquine in the second experiment, received the chloroquine 2 days earlier than the mother mice from the first experiment that were treated post partum. In this way, the chloroquine was already distributed to
their biological pups. So, part of the chloroquine was already metabolized and excreted, leading to lower chloroquine-concentrations in the switched litters. Additionally, the stress for the mother mice, caused by the switch of the pups and possibly resulting in poor nursing of the pups can also explain the higher death rate of the switched pups.

In an in vivo study of Barnard and colleagues, BALB/c mice were infected with SARS coronavirus and treated with chloroquine (6). In the study of Barnard et al no significant reduction in the replication of the SARS coronavirus in vitro and in vivo was seen, this in contrast with the findings of other published in vitro studies (16,34) and our in vivo study. This difference in findings is possibly due too a difference in animal model, an infection (non-lethal) BALB/c model versus a lethal newborn C57Bl/6 model and a different antiviral treatment scheme. Moreover, the in vitro antiviral effect of chloroquine against the SARS-CoV is less potent than the antiviral effect against HCoV-OC43 in vitro. For SARS-CoV, the plasma concentrations reached in mice may possibly be not high enough to protect against a SARS-CoV infection.

In conclusion, we here demonstrate that chloroquine shows a strong in vitro and in vivo antiviral activity against HCoV-OC43. Moreover, treatment with daily doses of chloroquine has a long-lasting protective effect against lethal coronavirus OC43 infection in newborn mice.
ACKNOWLEDGEMENTS

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REFERENCES


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\(^\d\)Absolute numbers of surviving animals 60 days after coronavirus OC43 challenge with between brackets the survival percentage. C57BL/6 mice were treated with 15, 5, 1 or 0 mg/kg chloroquine before challenge.

\(^\d\)ND: not done
**Figure Legends**

**Figure 1.** Time dependent antiviral effect of chloroquine on HCoV-OC43 replication in HRT-18 cells. HCoV-OC43-infected HRT-18 cells were treated with 10 µM chloroquine (CQ) at the time of infection or at different time points thereafter (●). The virus control represents HCoV-OC43 infected cells incubated with medium (■). The background (▼) represents the residual viral load, that was detected after washing the cells. Twenty-eight hours after infection, cell supernatants were collected and viral RNA was extracted and quantified by qRT-PCR. Data are mean values ± SEM of 2 independent experiments performed in duplicate.

**Figure 2.** Survival curves of newborn mice after HCoV-OC43 infection. Newborn mice were infected intracerebrally with $10^3$ HCoV-OC43 copies, and mother mice were treated subcutaneously with 1, 5 or 15 mg/kg chloroquine (CQ) pre (A) or post (B) partum. N represents the number of mother mice per group, n the number of newborn mice per group.

**Figure 3.** Survival curves of newborn mice after HCoV-OC43 infection. Five-day-old suckling mice were infected intracerebrally with $10^3$ HCoV-OC43 copies and did or did not receive chloroquine (CQ), either via the placenta or via the maternal milk. N represents the number of mother mice per group, n the number of newborn mice per group.
(A) 15 mg/kg CQ (n=70, N=9)
5 mg/kg CQ (n=42, N=5)
1 mg/kg CQ (n=21, N=4)
0 mg/kg CQ (n=132, N=19)

(B) 15 mg/kg CQ (n=76, N=11)
5 mg/kg CQ (n=42, N=6)
1 mg/kg CQ (n=31, N=4)
0 mg/kg CQ (n=132, N=19)
15 mg/kg CQ transplacentary (n=42, N=5)
0 mg/kg CQ (n=132, N=19)
15 mg/kg CQ via maternal milk (n=70, N=9)

% Survival

Time (days post infection)