Inter-species transmission of viral hemorrhagic septicemia virus (VHSV) from turbot (Scophthalmus maximus) to rainbow trout (Onchorhyncus mykiss)

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Successful viral infection is a complex mechanism, involving many host–pathogen interactions that developed during coevolution of host and pathogen, and often result in host-species specificity. Nevertheless, many viruses are able to infect several host species and sporadically cross species barriers. The viral hemorrhagic septicemia virus (VHSV), a rhabdovirus with high economic impact on the aquaculture industry, has developed an exceptionally wide host range across marine and freshwater environments. Transmission of VHSV between host species therefore represents a potential risk for aquaculture, which currently is not addressed in biosecurity managements. The objective of this study was to investigate the inter-species transmission potential of VHSV and evaluate whether infected marine wild fish pose a potential risk on marine cultured rainbow trout. A cohabitation infection trial with turbot as donor and rainbow trout as recipient host species was conducted. Turbot were intraperitoneally injected with either a marine-adapted (MA) or a trout-adapted (TA) VHSV isolate and subsequently grouped with naïve rainbow trout. Both VHSV isolates were able to replicate and cause mortality in turbot, while only the TA isolate was able to cross the species barrier and infect rainbow trout with fatal outcome. The results demonstrate that a marine fish species can function as reservoir and transmitter of TA VHSV isolates.

INTRODUCTION

Viruses are obligate intracellular pathogens that rely on host cells for replication. Successful viral infection is a complex mechanism involving many host–pathogen interactions. These interactions developed during coevolution of host and pathogen and often result in host-species specificity. Nevertheless, many viruses found in eukaryotic hosts are able to infect several host species and sporadically cross species barriers.

The viral hemorrhagic septicemia virus (VHSV) is an example of a viral species that has established an exceptionally wide host range of more than 80 teleost fish species known so far (Anonymous, 2009). VHSV is an enveloped negative-sense, ssRNA virus that belongs to the genus Novirhabdovirus within the family Rhabdoviridae. It is the causative agent of a serious disease in teleost fish with high impact on cultured fish species such as rainbow trout (Oncorynchus mykiss; Smail, 1999; Wolf, 1988), turbot (Scophthalmus maximus; Ross et al., 1995), and Japanese flounder (Paralichthys olivaceus; Isshiki et al., 2001) where it can cause mortality rates as high as 90 % (Olesen, 1998; Snow et al., 1999), posing a large economic threat to the aquaculture industry (Hill, 1992; Nylin & Olesen, 2001).

Four major genotypes have been identified (I, II, III and IV) with further subdivision of genotype I (Ia–Ie) and IV (IVa–IVc). The genotypes are related to geographical regions rather than host species or serotypes (Einer-Jensen et al., 2004; Snow et al., 1999). Genotypes I, II, and III are found in Europe, whereas genotype IV is found in North America, Korea and Japan (Benmansour et al., 1997; Einer-Jensen et al., 2004; Kim et al., 2011; Lumsden et al., 2007; Nishizawa et al., 2002; Snow et al., 1999; Stone et al., 1997).

Although the phylogeny does not directly reflect host range, experimental infections have demonstrated that European VHSV genotypes show different pathogenicity patterns depending on host species. European VHSV isolates originating from freshwater reared rainbow trout often belong to genotypes Ia, Ic, Id or Ie and are generally highly pathogenic to rainbow trout but show no or low pathogenicity to marine fish species. Correspondingly,
European VHSV isolates originating from marine fish species often belong to genotypes Ia, II or III and generally show no or very low pathogenicity to rainbow trout (Skall et al., 2004), dividing the European VHSV isolates into rainbow trout-adapted (TA) or non-adapted isolates, respectively. However, phylogenetic studies have revealed that TA isolates most likely evolved from a marine ancestor that managed to cross species barriers (Einer-Jensen et al., 2004). Recent outbreaks in marine cultured rainbow trout in Sweden (Nordblom, 1998; Nordblom & Norell, 2000), Finland (Husu-Kallio & Suokko, 2000; Raja-Halli et al., 2006) and Norway (Dale et al., 2012) support this hypothesis. Isolates originating from these outbreaks were genetically more closely related to isolates from marine species from the surrounding waters than to isolates from freshwater cultured rainbow trout (Dale et al., 2009; Einer-Jensen et al., 2004), indicating that inter-species transmission from marine to cultured species occasionally occurs (Kurath & Winton, 2011). The mechanisms or genetic background facilitating inter-species transmission of VHSV remain to be identified.

Transmission of VHSV is mainly established by horizontal transport of viral particles through the water (Kurath & Winton, 2011), by direct contact with infected fish, or by oral ingestion of infected material (Scho¨nherz Winton, 2011), or by transport of viral particles through the water (Kurath & Winton, 2011). The mechanisms or genetic background facilitating inter-species transmission of VHSV remain to be identified.

The objective of this study was to investigate the inter-species transmission potential of VHSV and to evaluate the potential risk posed to marine cultured rainbow trout by infected non-salmonid marine wild fish. An experimental cohabitation challenge with turbot as donor and rainbow trout as recipient host species was conducted using either a marine-adapted (MA; isolate DK-4p168) or a TA (isolate DK-3592B) VHSV isolate. Both viral isolates have recently been shown to be able to infect turbot by waterborne challenge (Snow et al., 2005; Stone et al., 1997) although the TA isolate showed very low pathogenicity under experimental conditions.

RESULTS

Mortality

Turbot were intraperitoneally (i.p.) injected with either the MA or the TA isolate and grouped together with naïve rainbow trout. Both viral treatments were conducted in duplicate with 10 i.p. injected turbot and 20±1 naïve rainbow trout per duplicate (Fig. 1). Cumulative mortality of both host species was recorded daily for the entire challenge period of 38 days (Fig. 2). Turbot exposed to the MA isolate initiated mortality at 14 days post-infection (p.i.) and reached a mean cumulative mortality of 100% (SD=0%). Cohabitating rainbow trout in these aquaria showed neither clinical signs nor mortality. Turbot exposed to the TA isolate initiated mortality at 23 days p.i. and reached a mean cumulative mortality of 45% (SD=19%; mortalityrep1=33%; mortalityrep2=60%). Cohabiting rainbow trout in these aquaria initiated mortality earlier than the i.p.-challenged turbot, as mortality among trout started 15 days p.i. and reached a mean cumulative mortality of 58% (SD=11%; mortalityrep1=65%; mortalityrep2=50%). The majority of fish that died during challenge showed clinical signs typical for infection with VHSV such as haemorrhages in eyes, oral cavity together with fin bases and darkening of the skin (Anonymous, 2009; Castric & de Kinkelin, 1984).

![Fig. 1. Schematic presentation of the viral challenge, indicating the two challenge phases, duration of challenge phases and total number of fish at the beginning of each phase. Dashed aquaria represent a non-challenged state. Dark grey coloration represents aquaria exposed to the MA isolate and light grey coloration represents aquaria exposed to the TA isolate. Twenty-four hours p.i. of turbot, they were distributed to aquaria containing naïve rainbow trout where they cohabited for 37 days. The experiment was terminated after 38 days p.i.](image-url)
Virological examination

Fish that died during the challenge period were individually subjected to virological examination using a cell culture assay, and all detected positive for VHSV, irrespective of host species or viral challenge isolate. Fish surviving the challenge period were also individually subjected to virological examination. All trout exposed to the MA isolate were negative for VHSV, whereas roughly half the trout surviving exposure to the TA isolate were positive for VHSV. Surviving turbot were only found in groups exposed to the TA isolate and roughly half of them were positive for VHSV. Results from virological examinations are summarized in Table 1.

Water sample analysis

Water samples were collected daily from day 1 to 18 following i.p. inoculation of turbot and analysed in duplicate by qRT-PCR to quantify shedding of virus. All water samples taken at 1 day p.i. were negative for VHSV, indicating that direct leakage of virus after injection of turbot was minimal. One single water sample tested positive at 2 days p.i., but could not be confirmed in the replicate sample and was considered to result from contamination in the qRT-PCR assay, since all other data were confirmed by synonymous results in the replicate samples.

In MA exposed groups, viral shedding was detectable at 4–5 days p.i. and was characterized by a steep increase in viral concentration to approximately $1 \times 10^3$ TCID$_{50}$ ml$^{-1}$ in the water, further increasing until 12–14 days p.i. with a peak around $4 \times 10^4$ TCID$_{50}$ ml$^{-1}$. Maximal viral concentrations coincided with initiation of mortality in turbot (Figs 2 and 3). Following onset of mortality, viral concentrations started to decline. In the TA exposed groups viral shedding started at the same time as in the MA exposed groups but increased more slowly to a peak of only $1 \times 10^3$ TCID$_{50}$ ml$^{-1}$ at 15 and 17 days p.i., followed by a subsequent moderate decline. On average, viral concentrations detected in the water of groups exposed to the TA isolate were 42-fold lower than concentrations detected in the water of groups exposed to the MA isolate. In the TA exposed groups, mortality in rainbow trout started as the virus concentration in the water peaked (15 days p.i.), whereas mortality among turbot started 8 days later at 23 days p.i. (Figs 2 and 3).

DISCUSSION

The pathogenicity of individual VHSV isolates has previously been considered host-specific, but recent outbreaks in Finland (Husu-Kallio & Suokko, 2000), Norway (Dale et al., 2009) and Sweden (Nordblom, 1998; Nordblom & Norell, 2000) indicated its ability to overcome species barriers and occasionally adapt to new hosts. Based on a MA and a rainbow TA VHSV isolate we have investigated its inter-species transmission potential; our results are, to the best of our knowledge, the first to demonstrate that turbot is able to transmit VHS disease to rainbow trout.

Inter-species transmission

For inter-species transmission to occur, the virus needs to establish full infection cycles in different host species,
including host entry, genome translation, replication, assembly of new functional virus particles and release into the environment. King et al. (2001) and Snow et al. (2005) demonstrated that both the MA isolate (68.6 and 48.4 % cumulative mortality, respectively) and the TA isolate (2.9 and 2.2 % cumulative mortality, respectively) can establish disease in turbot following a natural route of infection. Even though mortality rates following exposure to the TA isolate were very low, it should be realized that environmental conditions such as temperature, water quality, population density, and the occurrence of other infections or injuries are likely to affect these rates in natural environments. Given that both VHSV isolates can establish a successful infection in turbot (Snow et al., 2005; Stone et al., 1997) the present study aimed at determining whether cohabitation with infected turbot could lead to infection and clinical disease in rainbow trout. Virus shedding profiles in the water together with mortality in turbot and detection of viral particles in tissue samples confirmed the establishment of an active infection for both virus isolates.

Inter-species transmission was confirmed by outbreak of clinical disease in rainbow trout cohabitated with TA

<table>
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<tr>
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<th>MA isolate</th>
<th>TA isolate</th>
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<tr>
<td></td>
<td>Turbot</td>
<td>Rainbow trout</td>
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<td><strong>Fish that survived challenge</strong></td>
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<tr>
<td>Survivor/total (rep. 1; rep. 2)</td>
<td>0/20</td>
<td>38/39</td>
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<td>(0/10; 0/10)</td>
<td>(19/20; 19/19)</td>
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<td><strong>Fish that died during challenge</strong></td>
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<tr>
<td>Dead/total (rep. 1; rep. 2)</td>
<td>20/20</td>
<td>0/39</td>
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<tr>
<td></td>
<td>(10/10; 10/10)</td>
<td>(0/20; 0/19)</td>
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<tr>
<td><strong>Fish executed during challenge</strong></td>
<td></td>
<td></td>
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<tr>
<td>Executed/total (rep. 1; rep. 2)</td>
<td>0/20</td>
<td>1/39</td>
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<tr>
<td></td>
<td>(0/10; 0/10)</td>
<td>(1/20; 0/19)</td>
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<tr>
<td><strong>Fish detected VHSV positive</strong></td>
<td></td>
<td></td>
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<tr>
<td>Survivors positive/analysed</td>
<td>NA</td>
<td>0/38</td>
</tr>
<tr>
<td>Dead positive/analysed</td>
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<tr>
<td>Executed positive/analysed</td>
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**Table 1.** Virological examination of organ tissue from intraperitoneal (i.p.)-challenged turbot and cohabitating rainbow trout

The table summarizes total number of fish per treatment; number of dead, terminated and surviving individuals, together with the number of virologically examined and VHSV positive fish. Total numbers are based on both replicates. NA, Not applicable.
exposed turbot. Since no virus was detected in the water until 3 days p.i. of turbot, viral contamination, including leakage of viral particles following i.p. injection, was highly unlikely. Virus particles produced and shed by turbot thus were the source of infection in rainbow trout. Interestingly, cohabiting rainbow trout initiated mortality 8 days before turbot i.p. inoculated with the TA isolate, possibly reflecting higher virulence of this isolate to rainbow trout. Also, development of VHS disease in turbot, even with MA VHSV isolates, has been reported to require longer incubation periods (time between viral exposure and development of clinical symptoms and mortality) than has been observed in rainbow trout (Brudeseth et al., 2005). In contrast to the TA isolate, the MA isolate failed to establish a successful infection at a detectable level in rainbow trout. Since the fish were only examined at the termination time point (37 days of cohabitation) a transient low level of infection in rainbow trout cannot be excluded, but previous studies in rainbow trout demonstrated that i.p. challenge with the MA isolate failed to establish disease (Skall et al., 2004), suggesting limitations in the ability of the MA isolate to both enter and replicate in this host species. Taking this aspect into consideration virus particles found in the water of MA exposed aquaria most likely were produced by turbot only, whereas viral particles found in the water of TA exposed aquaria were assumed to have been produced initially by turbot and later by both turbot and rainbow trout. Interestingly, viral concentrations in water samples from TA-challenged fish were 42-fold lower than concentrations in corresponding water samples from aquaria with MA-challenged fish. This finding suggests that the latter virus isolate was secreted to a much higher degree than the TA isolate. Differences in viral production/secretion from an infected individual most likely evolved as a response to transmission rates. Viral transmission rates in cultured fish are generally high because of high stocking densities and intense production schemes, whereas in wild populations transmission might be limited by rapid dilution of shed virus and limited contact with susceptible hosts (Kurath & Winton, 2011). Accordingly, high viral production together with longer incubation periods, as observed for the MA isolate, would be of advantage for the virus to ensure more efficient transmission and thereby survival of the virus in marine environments.

Instead of being homogeneous, RNA virus populations form quasispecies, which are defined as a dynamic distribution of genomes subjected to genetic variation, competition and selection (Domingo et al., 1998). Each quasispecies is composed of a dominant nucleotide sequence and a mutant spectrum of low frequency variants (Domingo & Wain-Hobson, 2009; Domingo et al., 2012), enabling the virus population to adapt rapidly to environmental changes such as new hosts (Schneider & Roossinck, 2001). A high level of viral production/secretion should thus affect the composition and size of the quasispecies via the number and frequency of mutated particles. However, despite high virus secretion, the MA isolate here appeared unable to establish a successful infection in the cohabitating rainbow trout. Whether this can be attributed to qualitative or quantitative limitations of our experimental setup remains to be determined. Phylogenetic analysis nevertheless showed that TA VHSV isolates evolved from a marine ancestor (Einer-Jensen et al., 2004). Taking account of this evolutionary history, the TA isolate might still contain variants in the mutant spectrum that are adapted to the original hosts. Such a relationship has been found for foot-and-mouth disease virus (Ruiz-Jarabo et al., 2000). Alternatively, nucleotide substitutions acquired in ancestral hosts might be selectively neutral for replication in rainbow trout. A replication history of about 50 years in rainbow trout (Einer-Jensen et al., 2004) would probably have been too short to remove these substitutions by genetic drift and fully eliminate the ancestral capacity of the TA isolate to infect and replicate in turbot. Full genome sequence comparison of the TA isolate before and after passaging in turbot, including the quasispecies composition will be required to clarify these points.

Whatever the evolutionary mechanisms may be, the results presented demonstrate that marine fish species can support replication of TA VHSV strains and can act as viral carriers capable of transmitting VHSV back to rainbow trout. In terms of prophylaxis of such events, transfer of VHSV-infected rainbow trout to the sea should be avoided, since this would place the virus in close proximity to its historical host and potentially allow establishment of rainbow trout pathogenic VHSV infections in wild marine fish species.

**METHODS**

**Virus isolates.** Two genetically distinct VHSV isolates were designated MA and TA. These isolates were chosen based on their host origin and pathogenicity to rainbow trout. The MA isolate is a European marine isolate belonging to genotype III, which was originally isolated from Atlantic herring in the Skagerrak (VHSV DK-4p168) and characterized as non-pathogenic to rainbow trout by immersion challenge (Skall et al., 2004). The TA isolate is a European TA isolate belonging to genotype Ia, which was originally isolated from an outbreak in Danish cultured rainbow trout (VHSV DK-3592B) and characterized as highly pathogenic to rainbow trout by immersion challenge (Skall et al., 2004). Viral stocks were propagated on the bluegill fry caudal trunk cell line (BF-2; Wolf et al., 1966), titrated to quantify concentrations, and stored at −80 °C as described elsewhere (Schönherz et al., 2012).

**Host.** A total of 42 turbot (mean weight=17.6 g; SD=2.8 g) and 79 rainbow trout fingerling (mean weight=14.9 g; SD=2.2 g) were used for challenge. Rainbow trout eggs, obtained from Danish commercial fish farms, were disinfected, hatched and maintained in pathogen-free laboratory facilities at the National Veterinary Institute (VET), Aarhus, Denmark. Turbot were obtained at 3–4 g size from a commercial unit producing turbot juveniles for stocking (Veno Fish Farm) and maintained in sand-filtered UV-treated seawater at 33 % salinity. Before the challenge, fish were acclimatized for 11 days to aerated water of 15 % salinity and 9 ± 1 °C. Salinity and temperature were monitored daily and kept constant through acclimation and
challenge period. Aquaria were cleaned manually, and half of the water was renewed every second day. Fish feed on a commercial pellet diet ad libitum once a day.

**Virus challenge.** To analyse the inter-species transmission potential a cohabitation challenge with i.p.-injected turbot and naive cohabitating rainbow trout was established. The challenge was conducted in two phases: i.p. injection and inoculation of turbot; and cohabitation of inoculated turbot with naive rainbow trout (Fig. 1).

**Phase 1: i.p. injection and inoculation of turbot** Prior to inoculation turbot were starved for 24 h. Following anaesthesia in 0.01% benzocaine (ethyl p-aminobenzoate), the turbot were i.p. injected with 25 µl virus stock of 1 × 10^7 TCID50 ml^-1. Injected turbot were kept for 24 h in separate aquaria before being grouped together with rainbow trout. During those 24 h a high water circulation was established to wash out leaking virus.

**Phase 2: cohabitation** For each VHSV isolate, a total of 10 challenged turbot were moved to each of the two aquaria containing 20 ± 1 naive rainbow trout, establishing duplicates per treatment. Unhandled controls of both fish species were continuously monitored for development of irrelevant diseases or background mortality.

During the entire cohabitation experiment (38 days) fish were monitored, mortality was recorded and dead fish were removed daily. Removed fish were stored at −20 °C for subsequent virological examination. On a daily basis, 2 ml of water was sampled from each aquarium, starting 1 day p.i. shortly after cohabitation. Water samples were collected before water exchange and immediately stored at −20 °C for subsequent analysis by qRT-PCR to quantify the virus concentration as an indirect indicator for virus shedding. After 37 days of cohabitation the challenge was terminated and surviving fish were euthanized, frozen and examined virologically as described for dead fish.

**Virological examination.** All cohabiting rainbow trout, all surviving turbot and at least 50% of the turbot dying during challenge were subjected to virological examination. Fish were individually sampled for spleen, kidney, liver, heart and brain. Organ tissues were homogenized with a blender and sterile sand, resuspended in cell culture medium (1:1) with gentamicin (25 µg ml^-1) and incubated overnight at 4 °C. Subsequently, homogenates were centrifuged at 62,930 g for 15 min and serial dilutions of supernatants were prepared in cell culture medium. Supernatant from dead fish was diluted in fivefold dilutions (1 × 10^-1 to 1 × 10^-5) while supernatant from terminated survivors was diluted in threefold dilutions (1 × 3^-1 to 1 × 3^-3). For each dilution series, four replicates were inoculated onto BF-2 cells seeded in 96-well trays the day before (24 h), and then incubated at 15 °C for 7 days. The inoculated cell cultures were inspected for cytopathic effects (CPE) three times over an incubation period of 7 days. Samples that showed toxic effects or lack of CPE were recovered and subcultivated in fresh BF-2 cells for a further 7 days. Samples were classified as VHSV positive if CPE was detected in the absence of toxic effects, whereas samples were classified as VHSV negative if neither toxic effects nor CPE was detected after subcultivation.

**DNA extraction.** Total viral RNA was extracted from water samples collected daily at 1–18 days p.i. Water samples (2 ml) were ultracentrifuged for 1 h at 4 °C and 157,000 g. Supernatant was discarded and the remaining pellet was either kept at −80 °C or directly subjected to RNA extraction using the RNeasy Mini kit (Qiagen) according to the manufacturer’s protocol for extraction of RNA from cells (Qiagen). Total RNA was finally suspended in 50 µl RNase-free water (Qiagen), aliquoted (10 µl per aliquot) and stored at −80 °C.

**Reverse transcription and PCR (qRT-PCR).** To investigate viral replication and shedding, the concentration of VHSV in water was analysed using a one-step qRT-PCR (Jonstrup et al., 2013). Briefly, a single primer set, amplifying a 77 bp region within the ORF of the N-gene (sense 5’-AAACCTCCAGGATGTGCCTCC-3’; antisense 3’-TCTCGATCTCAGTGAGGTA-5’) was used for reverse transcription and subsequent qPCR in a single step to avoid contamination between steps. The qRT-PCR assay was performed on an MX Pro-Mx3005P thermocycler as described elsewhere (Sönherz et al., 2012). Briefly, reverse transcription to cDNA was conducted at 50 °C for 30 min and 95 °C for 15 min followed by PCR amplification of 40 cycles of denaturation (94 °C, 15 s), annealing (60 °C, 40 s) and extension (72 °C, 20 s) in a final volume of 25 µl containing 5 µl RNA template and 20 µl PCR master mix. The master mix was prepared from the QuantiTect Probe RT-PCR kit (Qiagen) containing 6.18 µl molecular graded water, 12.5 µl 2 × qRT-PCR master mix, 0.25 µl RT/RNase block enzyme mix, all supplied by the kit, 0.23 µl of each primer (100 pmol µl^-1) and 0.63 µl Taqman probe (per sample). To acquire absolute quantities 10-fold dilutions of the corresponding virus isolate with known concentration (MA isolate: 4 × 10^7 TCID50 ml^-1 to 4 × 10^9 TCID50 ml^-1; TA isolate: 5.9 × 10^7 TCID50 ml^-1 to 5.9 × 10^9 TCID50 ml^-1) were generated and used in each qRT-PCR run as standard curve. Negative controls included parallel amplification of RNase-free water with master mix or water only. All samples were analysed in duplicate.

**ACKNOWLEDGEMENTS**

This work was supported by grants from The Danish Agency for Science, Technology and Innovation (grant nos 09-066097 and 09-065033/FTP). The authors thank Hanne Buchholz and Lisbeth Troels for their excellent technical assistance, Torben Egil Kjær and Lene Nørskov for assistance during challenge trials, and Søren Peter Jonstrup for providing the qRT-PCR protocol and primers before the protocol was publicly available, as well as fruitful discussions regarding the analysis.

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