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A novel combination of TaqMan RT-PCR and a suspension microarray assay for the detection and species identification of pestiviruses

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Abstract

The genus Pestivirus contains four recognized species: classical swine fever virus, Border disease virus, bovine viral diarrhoea virus types 1 and 2. All are economically important and globally distributed but classical swine fever is the most serious, concerning losses and control measures. It affects both domestic pigs and wild boars. Outbreaks of this disease in domestic pigs call for the most serious measures of disease control, including a stamping out policy in Europe. Since all the members of the Pestivirus genus can infect swine, differential diagnosis using traditional methods poses some problems. Antibody tests may lack specificity due to cross-reactions, antigen capture ELISAs may have low sensitivity, and virus isolation may take several days or even longer time to complete. PCR-based tests overcome these problems for the most part, but in general lack the multiplexing capability to detect and differentiate all the pestiviruses simultaneously. The assay platform described here addresses all of these issues by combining the advantages of real-time PCR with the multiplexing capability of microarray technology. The platform includes a TaqMan real-time PCR designed for the universal detection of pestiviruses and a microarray assay that can use the amplicons produced in the real-time PCR to identify the specific pestivirus.

Keywords: pestivirus, CSFV, BDV, BVDV1, BVDV2, TaqMan real-time PCR, suspension microarray

1. Introduction

The genus Pestivirus in the family *Flaviviridae* includes economically important, widespread pathogens causing postnatal and prenatal infections in a range of host animals, such as cattle, swine, and sheep (Moennig & Plagemann, 1992). The recognized species are classical swine fever virus (CSFV), Border disease virus (BDV), and bovine viral diarrhoea (BVD) virus types 1 and 2 (BVDV1, BVDV2). Whereas CSFV appears to be restricted to swine and wild boars, BDV, BVDV1 and BVDV2 can infect various domestic and wild ungulate species, including swine (Becher et al., 2003). Cattle are susceptible for infections with both BVDV types and rarely with BDV, sheep can be infected with the two BVD viruses as well as BDV and pigs can be infected with CSFV, BDV, BVDV 1 and 2 (Deregt et al., 2006).

Pestiviruses are enveloped, positive sense, single-stranded RNA viruses with a genome of approximately 12 kb, which consists of one large open reading frame flanked by highly conserved 5' and 3' non-translated regions. They have closely related genomes and are antigenically cross-reactive, which can cause diagnostic uncertainty and errors (Hoffman et al., 2005). This is particularly relevant to the diagnosis of classical swine fever (CSF), where a false positive result may have severe economic consequences. CSF is on the list of diseases notifiable to the World Organization for Animal Health (OIE). In 1997, an outbreak of CSF in the Netherlands resulted in approximately 11 million pigs being put down and caused direct losses (excluding loss of exports) of over two billion U.S. dollars (Pluimers et al., 1999; Terpstra and de Smit, 2000). As evident by inspection of the data given by the OIE World Animal Health Information Database (WAHID),

pestivirus infections regularly emerge and re-emerge on all continents and improved differential diagnosis is an important requirement internationally.

BDV causes Border disease, a congenital disease affecting sheep and goats, although rare in goats. Clinical signs include abortions, barren ewes, stillbirths and small weak lambs, which can show tremors, abnormal body conformation and abnormal birthcoat (Nettleton et al., 1998).

BVDV is endemic in most cattle-raising countries globally, with 60–85% of adult cattle being antibody positive (Houe, 1999). The outcome of infection of susceptible females with BVDV includes embryonic death, abortion, foetal anomalies and/or the birth of persistently infected (PI) animals. After birth, BVDV may cause a range of disease syndromes and might also have immunosuppressive potential, which can result in increased susceptibility and more severe clinical effects of other infectious agents (Potgieter, 1995).

Recent progress in real-time PCR-based diagnostics has been reported employing a variety of novel molecular chemistries including molecular beacons (McKillen et al., 2006) and primer-probe energy transfer (Hakhverdyan et al., 2006) with the attempt to improve factors such as specificity, multiplexing and robustness towards mutation in the probe region. However, in real-time PCR the number of parallel detections are limited by the number of available fluorescence channels on the real-time fluorescence reader (to our knowledge at most six at the time of writing) and by difficulties caused by cross-talk between the fluorescence channels and interactions between simultaneously amplifying PCR and for these reasons rarely exceeds four or five (Vet et al., 1999; Molenkamp et al., 2007). In this paper, we describe an assay platform that combines general TaqMan real-

time PCR for optimized high sensitivity detection of pestiviruses with a low-density microarray for subsequent species identification. The assay is well suited for implementation in a routine diagnostic laboratory for the screening of clinical samples as well as confirming and identifying virus isolates in culture.

2. Materials and Methods

2.1 Sample preparations

The quantitated pestivirus RNA samples used in the present study were taken from a panel of pestivirus strains and isolates supplied by The Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Insel Riems, Germany (Table 1). This panel of pestiviruses represented a collection of CSFV, BDV, BVDV1 and BVDV2, which was pre-tested by the EPIZONE international consortium, thus, it provided a reliable basis for the estimation of pestivirus detection capacity. Samples were provided with a concentration of 10^4 copies/µl. All experiments, excluding sensitivity tests, were performed using samples that were diluted 100 times in RNase-free water unless otherwise mentioned and used in the PCR experiments without further purification. In addition, one isolate, representing a recently discovered atypical bovine pestivirus, was provided by our laboratories at the Swedish National Veterinary Institute (SVA). This isolate, originating from cattle in Thailand, was currently referred to as *TKK virus* (Ståhl et al., 2007; Liu et al., 2008).

Sensitivity was estimated using a dilutions series of the *Alfort 187 1.1* CSFV strain from the pestivirus panel. Specificity tests were performed using RNA extracted

from four swine influenza isolates propagated in eggs. Cultured samples of bluetongue virus subtypes 2 and 8, porcine herpesvirus type 1 and bovine herpesvirus type 1 were also used to test specificity. Extractions were performed by Trizol-chloroform method according to manufacturer's instructions (Invitrogen). Negative serum, intestine and semen samples from swine and negative blood samples from cattle were also tested. For these samples the RNA was extracted using the GenoM-48 workstation (GenoVision/Qiagen) and the MagAttract Virus Mini M48 kit (Qiagen). 400 µl of sample material was extracted according to MagAttract Virus Mini Protocol version 0.5.2 and eluted in 100µl RNase free water containing 0.04% sodium azide.

2.2 Reverse transcription and real-time PCR

Three sets of previously published primers, all from the conserved 5'-UTR region, were evaluated on the entire set of panel samples. The primers were those published by Vilcek et al (1994): forward primer, ATG CCC (T/A)TA GTA GGA CTA GCA ; reverse primer, TCA ACT CCA TGT GCC ATG TAC, by Elvander et al. (1998): forward primer, GCT AGC CAT GCC CTT AGT AGG A; reverse primer, ATC AAC TCC ATG TGC CAT GTA CAG C, and by Deregt et al. (2006): forward primer, CAT GCC C(A/G)(C/T) AGT AGG ACT AGC; reverse primer, ATG TGC CAT GTA CAG CAG AG. In the followings, the primer pairs published in the above listed three articles will be referred to by **Vil**, **Elv** and **Der**, respectively. It has been shown that asymmetric PCR, which creates an excess of one of the strands, yields better signal-to-noise on Luminex equipment (Deregt et al., 2006). In the present study we take a different approach and use Lambda exonuclease to digest one of the amplicon strands. To facilitate

the digestion, the forward primers were 5´-phosphorylated. In addition the reverse primers were 5'-biotinylated to allow conjugation of streptavidin-phycoerythrin for later detection by the Luminex instrument. The real-time probe construct was as follows: 5´ 6-Fam-TGG GCA TGC CCT CGT CCA C-BHQ-1 3´. This probe was designed, validated and is currently in use at the routine diagnostic laboratory of the Swedish National Veterinary Institute (P. Thorén, SVA, personal communication). One-step RT-PCR reactions were employed using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq from Invitrogen. The reaction volume was 12.5 µl and the primer and probe concentrations were 1.3 and 0.25 µM, respectively. The PCR cycling profile was as follows: a reverse transcription step at 50°C for 30 minutes, an enzyme activation step at 94°C for 2 minutes and, finally, 50 PCR cycles with 15 seconds denaturation at 95°C, 15 seconds primer annealing at 53°C and 40 seconds elongation at 72°C. Fluorescence was read during the annealing phase. PCR was performed on a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia). To carry out the nuclease digestion 0.15 µl Lambda exonuclease (Fermentas Life Sciences, Burlington, Canada) was added to the PCR mixture. The single strand product was generated by incubating the samples at 37°C for 30 minutes, followed by an enzyme inactivation step of 15 minutes at 80°C.

2.3 Suspension microarray

Eight amino-functionalised ssDNA oligonucleotides (Biomers) ranging from 18 to 45 bases were selected from a previous study (Deregt et al., 2006) as capture probes for the array. Species specific probes included two universal CSFV probes (CSFV A and CSFV B) and one each for BVDV 1, BVDV 2, and BDV. Also, one universal BVDV

probe (BVDV Com) and two universal pestivirus probes (Pesti Com A and Pesti Com B) were used. A ninth probe with the sequence: 5´-CGA CGC ATC AAG GAA TGC CTC GAG was added for the detection of the atypical bovine pestivirus *TKK*. The oligonucleotides were coupled to carboxylated microspheres (Luminex Corporation, Austin, TX, USA) according to the protocol recommended by the manufacturer.

The bead stocks (50,000 beads/µl) were diluted in 1.5× TMAC hybridization buffer such that 5,000 of each probe bead were present in each sample well. Hybridization reactions were carried out in a total volume of 50 µl/well consisting of 5 µl of the PCR product, 10 µl TE buffer and 35 µl of the bead mix in duplicate in 96-well microtiter plates (Fisher Scientific, Waltham, MA, USA).. For a background control, a no template control from the PCR assay was used. The microtiter plates were incubated on a shaker plate at 50°C for 30 minutes at 600 rpm. A 12 µl volume of streptavidin- R-phycoerythrin (Caltag Laboratories, Burlingame, CA, USA) in 1× TMAC hybridization buffer, at a concentration of 0.01 mg/ml, was added and mixed with the reactants and incubation was continued at 50°C for 15 min. The median fluorescent intensity (MFI) of each reaction was measured in Luminex LX200 instrument at 50°C. A minimum of one hundred beads per probe was analyzed to calculate the MFI of each reaction.

3. Results and Discussion

3.1 Primer evaluation

All three primer pairs performed competently, successfully amplified the target viruses in the majority of samples, however, the **Elv** primers failed to amplify CSFV

185 *Congenital Tremor (3.1)* and BDV *137/4 (1)* at 10 000 copies, *Congenital Tremor 3.1*
186 amplified but poorly and *137/4 (1)* failed six out of seven times, amplifying very poorly
187 the one time it was successful (data not shown).

188 In initial tests the **Vil** and **Der** primer combinations amplified all target viruses.
189 Both primers sets were less effective compared with the other strains, for these three
190 pestivirus strains: CSFV *Congenital Tremor (3.1)*, BDV *Gifhorn (3)* and BDV *137/4 (1)*.
191 The **Der** primers proved, however, more robust over a broad range of primer
192 concentrations during the optimization experiments and at optimized conditions never
193 failed to amplify these strains while the **Vil** primers failed 44% of the time even at
194 optimal primer concentration. A failure considered a low fluorescence (< 10:1 signal to
195 noise with NTC) or a Ct > 35. Figure 1 shows results from one experiment using the **Vil**
196 (empty symbols) and **Der** (filled symbols) primer pairs for amplification of 100 viral
197 genome copies of the *CSFV Congenital Tremor (3.1)*, BDV *137/4 (1)* and BDV *Gifhorn*
198 *(3)* strains. All amplifications were detected with the same TaqMan probe (see Materials
199 and Methods). It is seen that the fluorescence signals appear 2-5 cycles earlier with the
200 **Der** primers and yield higher signal than with the **Vil** primers. Similar results were
201 obtained for other experiments with these strains (data not shown) and changes of primer
202 ratios or concentrations could not alter this general pattern. For these reasons the **Der**
203 primers where chosen for further experiments.
204
205 *3.2 Real-time detection followed by identification on suspension microarray*
206 All 24 pestivirus strains and isolates (Table 1), except the *TKK isolate*, were run
207 in duplicate in a single experiment on a RotorGene 6000 real-time PCR instrument. For

ease of viewing the duplicates have been averaged and the amplification curves are shown in Figure 2 in three different panels: panel A (CSFV); panel C (BDV); panel E (BVDV1, BVDV2 and *TKK*). The *TKK* amplification curve, likewise displayed as the average of a duplicate run, was obtained in a separate experiment but with the same temperature cycle. All strains were readily detected at the level of 100 genome copies per reaction with the **Der** primers (Figure 2, panels A, C & E); hence the detection rate in this study was 100%. In accordance with the fact that all samples were pre-quantified, all strains belonging to the same species (CSFV, BDV, BVDV1 or BVDV2) appeared at a similar cycle threshold (Ct) with the sole exception of the *Congenital Tremor 3.1* strain that appeared 2-3 cycles later. This observation corroborates the generally observed greater difficulty to detect this sample as compared to the other strains investigated in this study. The late Ct value of the *TKK isolate* is not remarkable since the genome equivalents of this isolate were not precisely quantified in the present study. No amplification could be traced in the "no template" control samples (Figure 2A and 2E).

In the microarray experiments, all nine probes generated a similar signal for no template control samples with an average MFI (median fluorescence intensity) of 38 and a standard deviation of 12. The threshold MFI signifying a pestivirus positive sample was set to 500 corresponding to ten times the sum of the average background and one standard deviation. The cross-species specificity was good as well with an average MFI of 43, only slightly higher than observed for the non-template control and a standard deviation of 33. The higher standard deviation originates from a slight interaction between the BDV probe with some other species isolates and a minor cross-reaction between the types 1 and type 2 BVDV specific probes. The strongest cross-species signal

was well below the threshold level at 281 MFI and observed for the BVDV 2 probe with the BVDV 1 *NADL* strain. Using the threshold at 500 MFI, indicated with a horizontal line in panels B, D and F of Figure 2, all samples were correctly identified by their virus species except for the BDV *Isard (2)* that fell below the threshold level with a MFI value of 285. However, since the real-time PCR data clearly shows the presence of a pestivirus for this sample the weak signal could be taken as evidence for the presence of a border disease virus or warrant further investigations of the nature of the virus species. This highlights the strength of the combination of real-time PCR with the Luminex typing. The PCR product of the atypical bovine pestivirus, *TKK*, did not cross-react with any of the other species specific and no cross-reactivity was observed between the new *TKK* probe and the recognized pestivirus strains (Figure 2).

Considering the positive signals in the microarray experiments it is clear that the common pestivirus probe A is less efficient than the B variant (Figure 2). The A probe frequently produces signals close to the threshold, e.g. the MFI for the CSFV strains: *C-Stamm (1.1), Alfort 187 (1.1), Brescia (1.2), Schweiz II (1.2), D4886/82/Ro (2.2)* the MFI's are 467, 737, 627, 573 and 541, respectively, and for the CSFV *Kanagawa (3.4)* strain the probe even failed with an MFI of 217 (see Figure 2 and supplementary information). In addition, for the BDV strains, the common pestivirus probe A failed in three of four cases (Figure 2D). The common pestivirus probe B usually produce a stronger signal and detects all strains from the pestivirus panel, however, the novel atypical bovine pestivirus *TKK* was not detected by this probe (Figure 2F). The significance of the probe redundancy used by Deregt et al. (2006) is illustrated by this observation; the two common pestivirus probes together detect all 24 samples while

neither of the probes could do that alone. In common with Deregt et al. (2006) we observe (Figure 2B) that the species specific CSFV probe A are superior to the CSFV probe B that fails to detect five strains (*Schweiz II (1.2), Paderborn (1.2), Bergen (2.2), D4886/82/Ro (2.2)* and *Uelzen (2.3)*) and detects a forth strain weakly (*Spante (2.3)*). However, it is interesting to note that, in our hands, the CSVF B probe is specific for genotype 1 and 3 while genotype 2 is not detected by this probe or detected weakly (Figure 2B). Hence, the combination of CSFV probes A and B provides some genotyping capacity. As mentioned above, the Luminex BDV probe detects three of four BDV strains. Finally, the common BVDV probe successfully detects all six strain of BVDV in the pestivirus panel and the type specific probes detect type 1 or 2 exclusively. However, none of the three BVDV probes produced a signal from the PCR product of the atypical bovine pestivirus *TKK* that only could be observed with the *TKK* specific probe (Figure 2F).

3.3 Sensitivity and specificity

In view of the similarity of the cycle threshold values of all pre-quantified samples from the pestivirus panel we only carried out sensitivity determinations on the CSFV strain *Alfort 187 (1.1)*. Since the CSFV strain *Congenital Tremor (3.1)* (Figure 2A) and the BVDV 2 strains (Figure 2E) appeared later by 2-3 cycles than what was observed for the other strains it can be anticipated that the sensitivity estimate obtained with *Alfort 187 (1.1)* is an upper limit for these strains. In panel A of Figure 3 the real-time PCR results of a serial dilution of the *Alfort 187 (1.1)* strain is shown and in panel B the subsequent Luminex suspension microarray data obtained with the common pestivirus

277 probes (circles) and specific CSFV probes (squares) are shown. The real-time data
278 represent the average of duplicate or triplicate runs. The detection sensitivity as revealed
279 by the PCR experiment is 6.4 viral genome copies/reaction. Correspondingly, the
280 Luminex signals are above the threshold with all four probes down to the 6.4
281 copies/reaction. The practical utility of this observation is that samples negative in the
282 real-time PCR experiment do not need to be run in the suspension microarray experiment.
283 Specificity tests revealed that none of the heterologous viruses and clinical
284 negative samples listed in section 2.1 were detected in real-time or on the microarray
285 (data not shown).
286
287 **4. Conclusions**
288
289 In the present study we have demonstrated the utility of combining a TaqMan
290 principle based real-time PCR detection with Luminex typing of pestiviruses. To
291 maintain optimized condition in the TaqMan amplification, asymmetric PCR was not
292 used to generate the single-strand target for subsequent Luminex experiments as
293 previously reported (Deregt et al. 2006); Lambda exonuclease was used instead. The real-
294 time PCR and the Luminex experiments had almost identical sensitivity, with the only
295 difference being the appearance of an amplification curve for a BDV isolate that failed to
296 reach the threshold level in the Luminex experiment. However, even in that instance the
297 signal for BDV on the Luminex was more than 4 times higher than for the other specific
298 pestivirus probes, which provided a good indication of a BDV positive sample. Given
299 this correlation between the real-time PCR and Luminex results, the real-time PCR can

function as a gateway where samples can be deemed negative or passed on for typing by Luminex experiments. The TaqMan real-time PCR also gives a quantitative readout with a wide dynamic range, which can be useful for research purposes. We also readily extended the microarray probe set to include a novel atypical bovine pestivirus isolate, *TKK* (Ståhl et al., 2007). The usefulness of redundancy in the Luminex probe set was noted since none of the two common pestivirus probes detected the respective viruses in all 24 samples but results from the two probes combined managed to do so. These observations illustrate the strength of the low density suspension microarrays coupled with real-time single-plex detection as compared to conventional PCR-based multiplex techniques; redundancy can be introduced to make the assay more robust and the assay format is easily extensible to include novel or emerging pathogens. The strains and isolates used in this study were obtained from the European Network of Excellence, EPIZONE, 2007 pestivirus ring trial, confirming the validity of this platform for clinically important variants of the four pestiviruses.

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Conflict of Interest Statement

There is no conflict of interest to declare in regard to this manuscript.

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405

Table 1
Pestivirus panel strains[a] and isolate(s)[b]

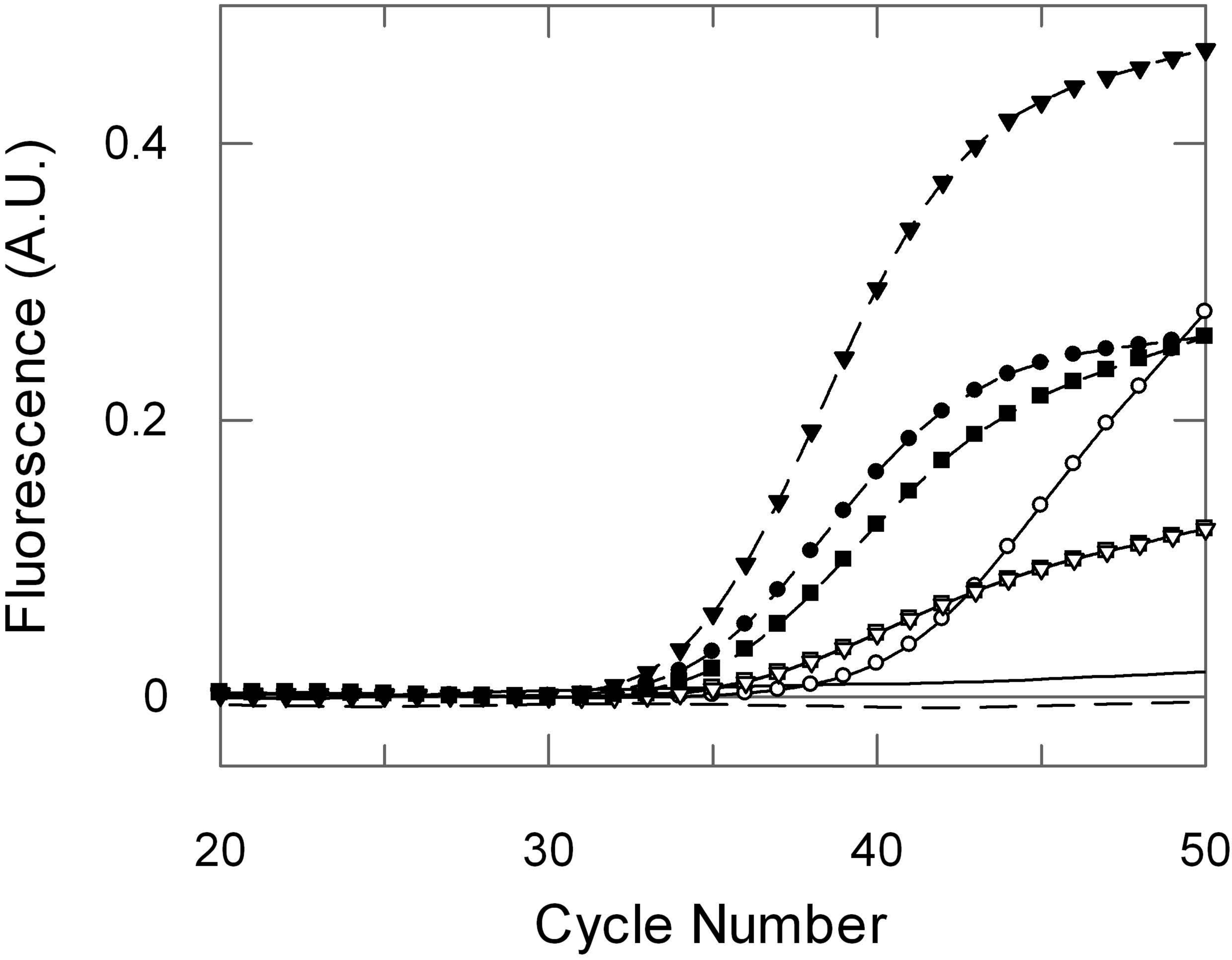
CSFV	BVDV	BDV
C-Stamm (1.1)	*Type 1*	Gifhorn (3)
Eystrup91 (1.1)	cp7	137/4 (1)
Alfort 187 (1.1)	NADL	Rudolph (2)
Koslov1128 (1.2)	Grub	Isard (4)
Brescia (1.2)	*Type 2*	
Schweiz II (1.2)	München	
Pader (2.1)	CS8644	
Bergen (2.2)	Bure	
D4886/82/Ro (2.2)	*Atypical bovine pestivirus isolate*[b]	
Uelzen (2.3)	TKK	
Spante (2.3)		
Congenital Tremor (3.1)		
Kanagawa (3.4)		

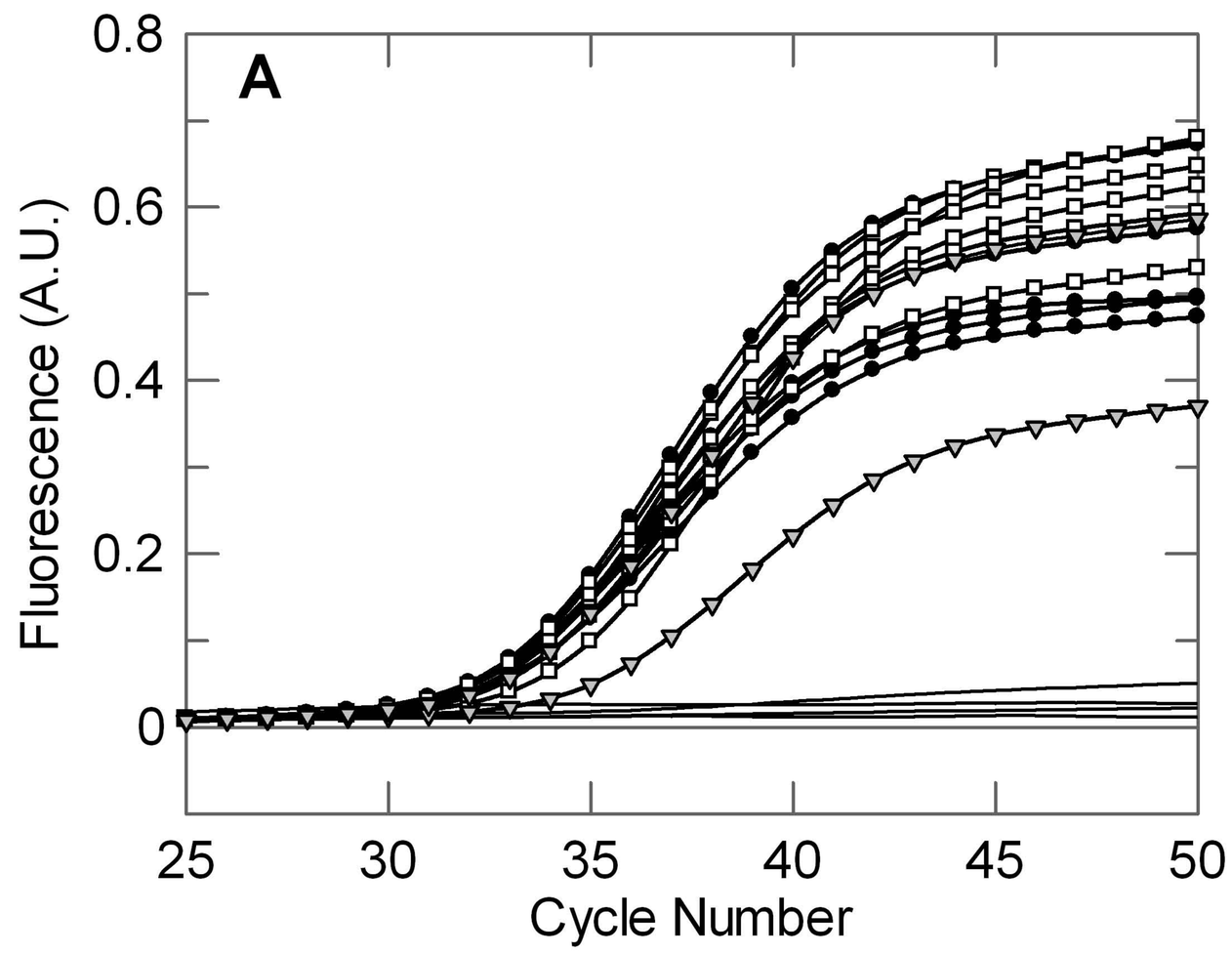
[a]Supplied by The Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Insel Riems, Germany except where indicated.
[b]From the virus collection of the Joint R&D Division in Virology of the National Veterinary Institute and of the Swedish University of Agricultural Sciences , Uppsala, Sweden

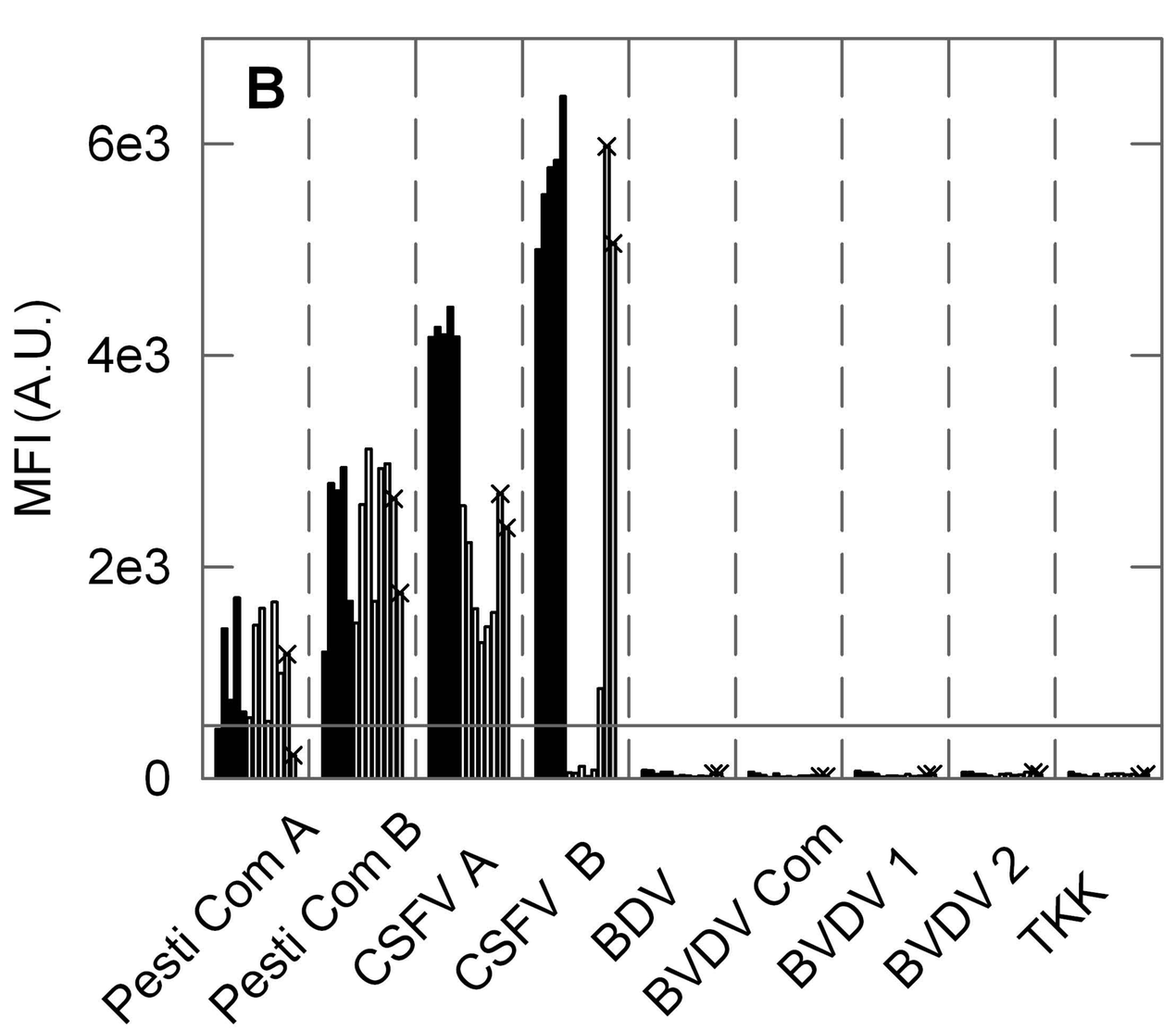
406

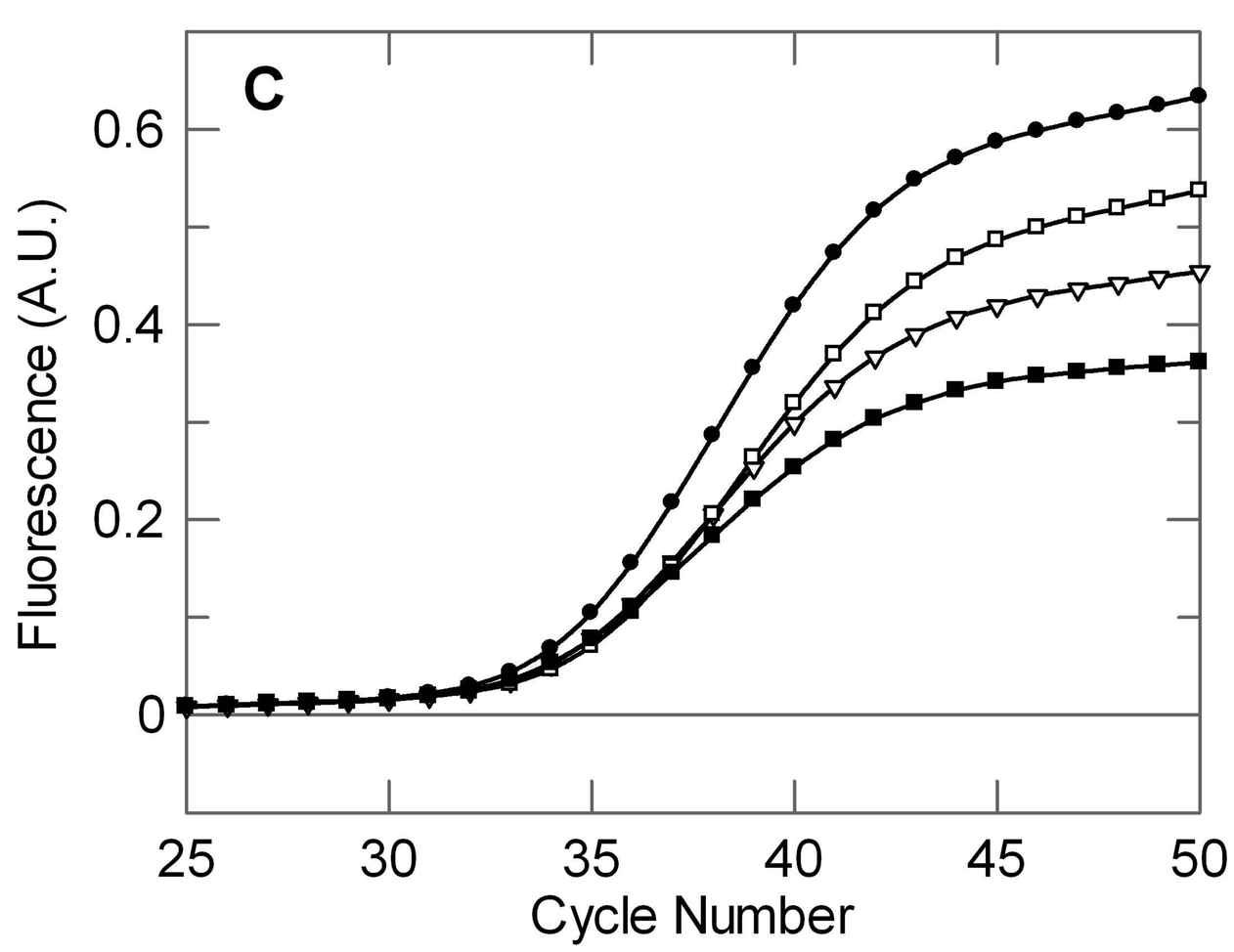
406 **Figure Legends**

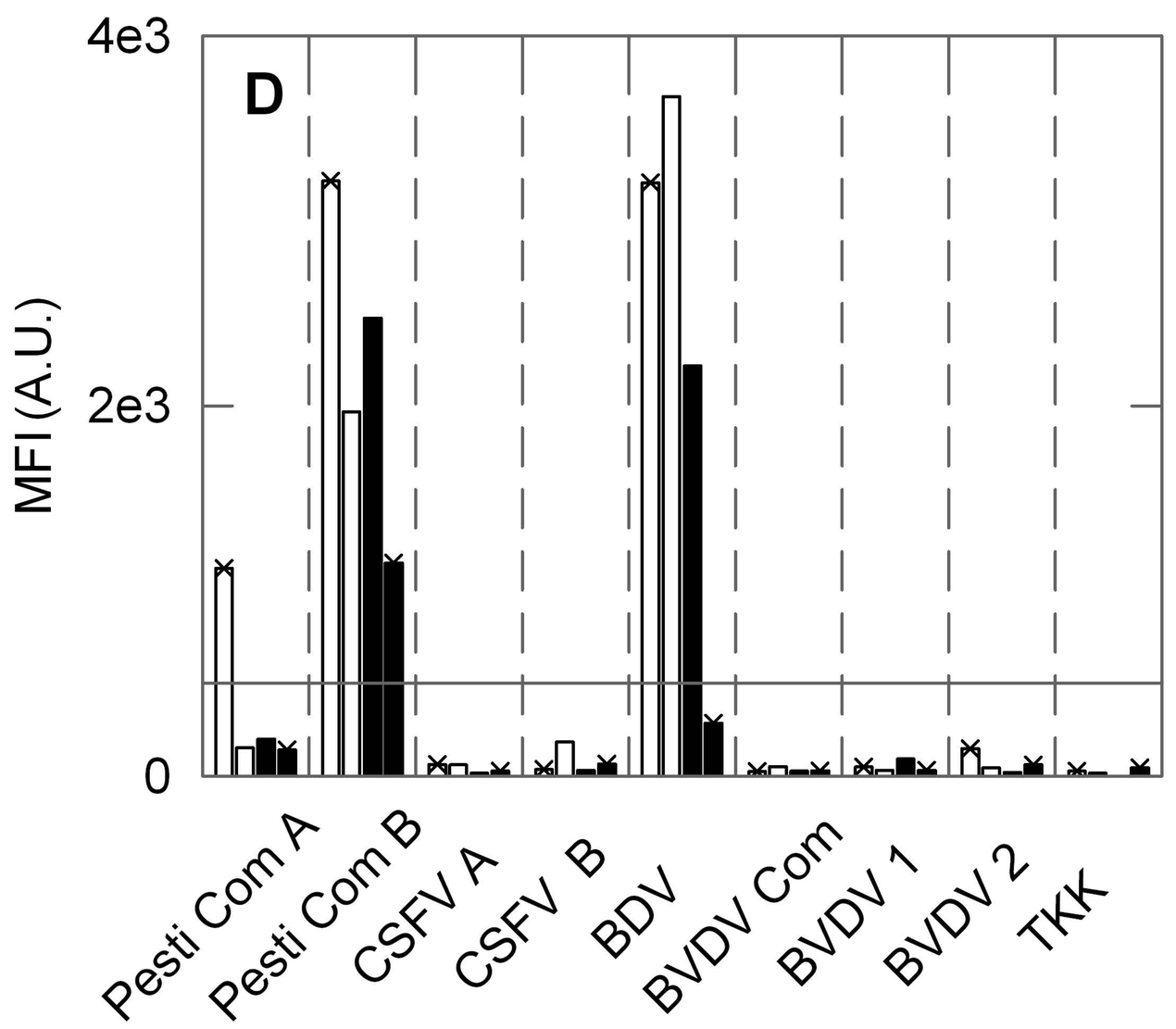
407 **Figure 1:** TaqMan real-time amplification curves obtained using the **Der** primers (filled
408 symbols) and the **Vil** primers (empty symbols) with the CSFV strain *Congenital Tremor*
409 *3.1* (●, ○) and the BDV strains *Gifhorn 3* (■, □) and *137/4 1* (▼, ▽) used as
410 templates. Each fluorescence value represents the average of a duplicate experiment.
411
412 **Figure 2:** The TaqMan real-time PCR data obtained for the (A) CSFV strains of
413 genotype 1 (●), 2 (□) and 3 (▽); (C) BDV strains of genotype 1 (□), 2 (●), 3 (▽) and
414 4 (■); and (E) BVDV type 1 (●), type 2 (□) and the atypical *TKK isolate* (▽). Four
415 non-template controls in the real-time PCR run are displayed as solid lines in panel (A)
416 and two corresponding to the *TKK* run in panel (E). The corresponding Luminex median
417 fluorescence intensity data (MFI) for (B) CSFV strains of genotype 1 (filled bars), 2
418 (empty bars) and 3 (empty bars with "×"); (D) BDV strains of genotype 1 (empty bars), 2
419 (filled bars), 3 (filled bars with "×") and 4 (empty bars with "×"); and (F) BVDV type 1
420 (filled bars), type 2 (empty bars) and the atypical *TKK isolate* (empty bars with "×"). The
421 threshold level of 500 MFI is displayed as a horizontal line in panels (B, D, F).
422 Information about how the fluorescence values were obtained can be found in the text.
423
424 **Figure 3:** (A) The real-time PCR data of a serial dilution of the CSFV strain Alfort 187
425 (1.1). The copy numbers for each dilution step are indicated. Two no-template controls
426 are displayed as solid lines. (B) The corresponding Luminex MFI values obtained with
427 the common pestivirus probe A (○) and B (●) and the CSFV A (□) and CSFV B (■)
428 probes. The threshold level of 500 MFI has been indicated with a horizontal line.

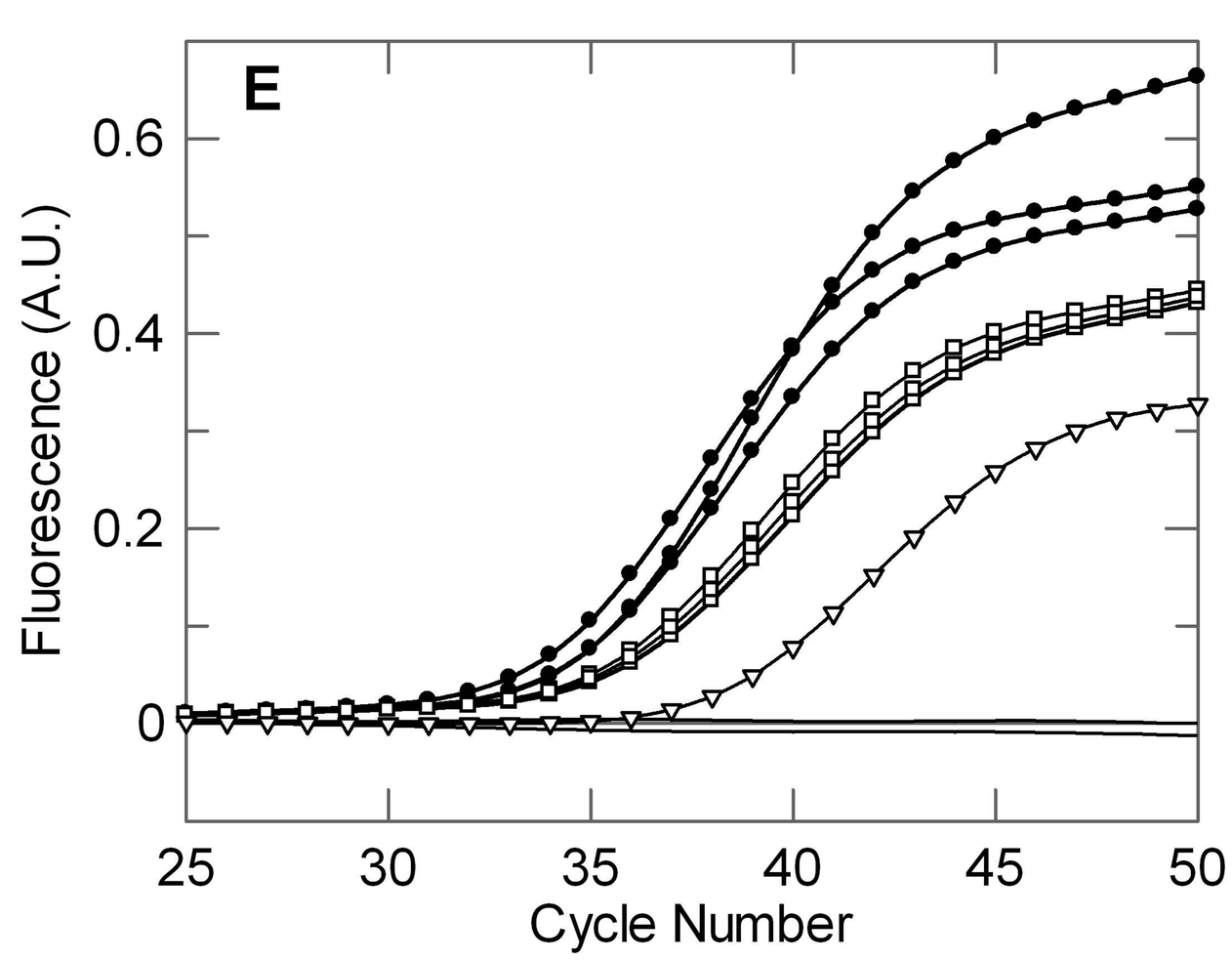

Fluorescence (A.U.)
0.4
0.2
0
20
30
40
50
Cycle Number

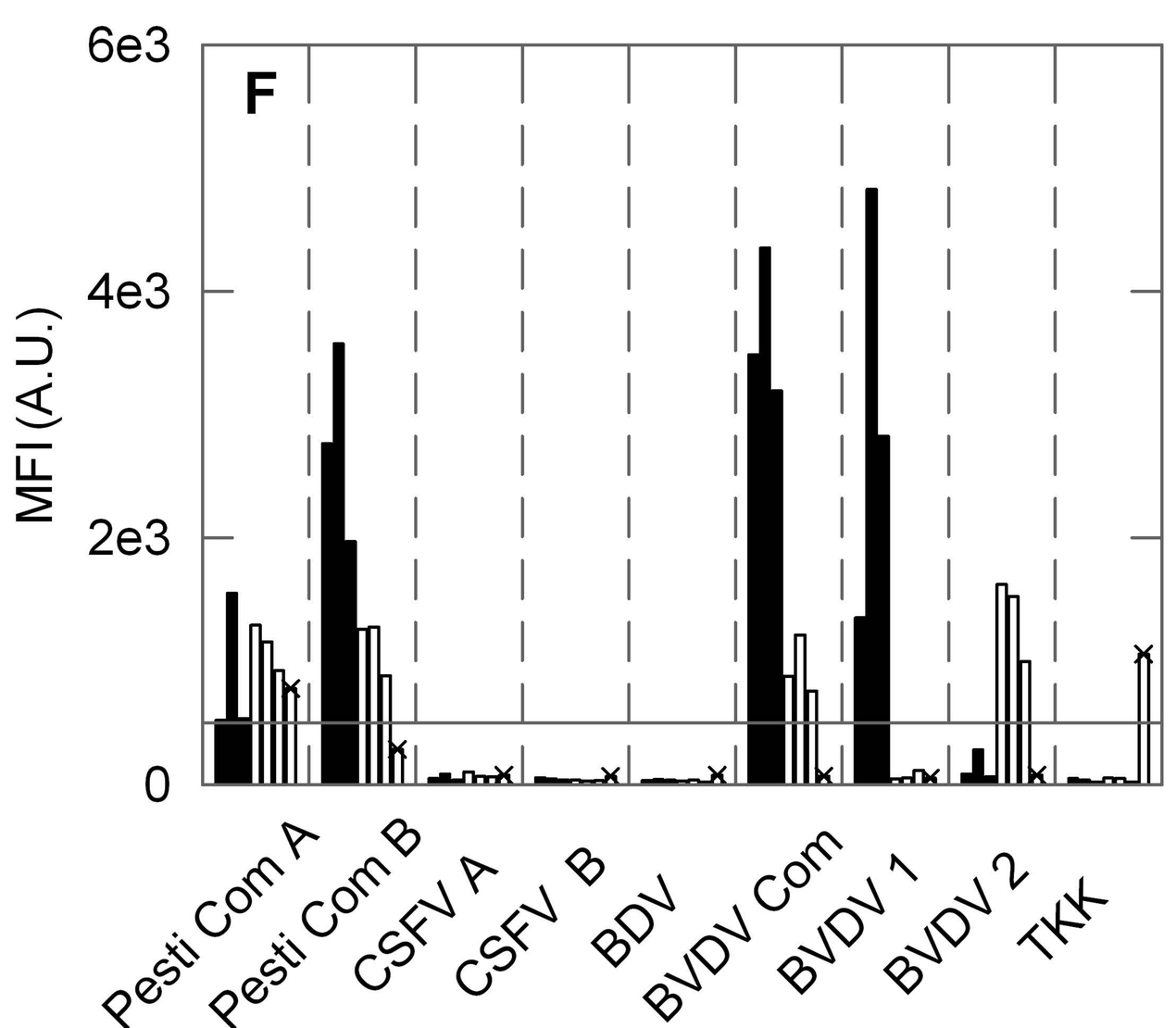

A
Fluorescence (A.U.)
0.8
0.6
0.4
0.2
0
25
30
35
40
45
50
Cycle Number


B
MFI (A.U.)
6e3
4e3
2e3
0
Pesti Com A
Pesti Com B
CSFV A
CSFV B
BDV
BVDV Com
BVDV 1
BVDV 2
TKK


C
Fluorescence (A.U.)
0.6
0.4
0.2
0
25
30
35
40
45
50
Cycle Number


D
MFI (A.U.)
4e3
2e3
0
Pesti Com A
Pesti Com B
CSFV A
CSFV B
BDV
BVDV Com
BVDV 1
BVDV 2
TKK


E
Fluorescence (A.U.)
0.6
0.4
0.2
0
25
30
35
40
45
50
Cycle Number


F
MFI (A.U.)
6e3
4e3
2e3
0
Pesti Com A
Pesti Com B
CSFV A
CSFV B
BDV
BVDV Com
BVDV 1
BVDV 2
TKK

A

Normalized Fluorescence (A.U.)

0.6

0.4

0.2

0

4000

800

160

32

6.4

1.28

20

30

40

50

Cycle number

B

MFI (A.U.)

6000

5000

4000

3000

2000

1000

0

4000

800

160

32

6.4

1.28

0.256

Copies/µl