Nictitating Membrane as a Potentially Useful Postmortem Diagnostic Specimen for Classical Swine Fever

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BRIEF COMMUNICATIONS

Nictitating membrane as a potentially useful postmortem diagnostic specimen for classical swine fever

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Abstract. The gold standard for diagnosis of classical swine fever (CSF) is cell culture virus isolation combined with reverse transcriptase–polymerase chain reaction (RT-PCR) and fluorescent antibody test (FAT) in cryosections of tonsils, spleen, various lymph nodes, ileum, and kidney. Autolytic and heterolytic samples render correct FAT evaluation difficult and can even yield false-negative or ambiguously positive results. To extend the spectrum of CSF diagnostic specimens, the authors tested whether the nictitating membrane (NM) might be a useful adjunct diagnostic specimen in wild boars and domestic pigs. To accomplish this, results of virus isolation, FAT, and RT-PCR were compared on NM samples and lymphoid tissues, which are the routine specimens of choice for CSF diagnosis. Wild boars ($n = 30$) and domestic pigs ($n = 8$) were experimentally challenged with various CSF virus (CSFV) strains or isolates of different virulence. The FAT revealed CSFV antigen in surface and tubular adenoid epithelium as well as in lymphatic follicles of the NM. In wild boars and domestic pigs with CSF, a strong agreement was found between results of FAT, virus isolation, and RT-PCR on NM and lymphoid tissues. These results suggest that NM is a useful additional specimen that can provide valuable data for postmortem diagnosis of CSF. The NM is relatively easy to sample at necropsy, and postmortem autolysis and heterolysis of this tissue is minimal compared with internal organs.

Key words: Classical swine fever; fluorescent antibody test; hog cholera; nictitating membrane; wild boars.

Classical swine fever (CSF, hog cholera) is a highly contagious systemic disease of domestic pigs and wild boars. Because of the severe economic losses it causes, control of this OIE ''List A'' disease is of worldwide interest. The CSF is caused by several variably virulent strains of a 40–50 nm, enveloped, single-stranded, positive-sense RNA virus, CSF virus (CSFV), which is a member of the Flaviviridae family, genus *Pestivirus*.[6,10,18]

In Germany, the presence of CSF in wild boars has been known to be responsible for epizootic spread to domestic pigs since the mid 1980s. The clinical signs and gross lesions observed at necropsy can vary considerably between domestic pigs and wild boars, and most of the lesions lack sufficient specificity for definitive diagnosis. In wild boars, the clinicopathology of CSF is more difficult to follow because hemorrhages do not occur regularly or are extremely difficult to observe, making the clinical and postmortem diagnosis of CSF a challenge for hunters and veterinarians. Therefore, CSFV in wild boars is best diagnosed in a holistic way by collecting clinical data and performing a variety of laboratory tests (virus isolation, reverse transcriptase–polymerase chain reaction [RT-PCR], fluorescent antibody staining of viral antigen in cryosections, CSFV-specific serology). Lymphoid tissues, which are primary targets of CSFV, are the best target samples for diagnosis. Proper sampling of fresh tissues from tonsils, various lymph nodes, spleen, and kidney that are suitable for fluorescent antibody test (FAT) is often difficult or impossible because of postmortem alterations. The CSFV infects and replicates in a broad range of tissues but affects primarily the immune system, resulting in a generalized leukopenia.[2,3] Circulating peripheral blood monocytes, lymphocytes, and macrophages of lymphoid organs and other tissues, including osteoblasts and progenitor cells of bone marrow, endothelia, and epithelial cells of lungs, kidneys, gall bladder, and tonsils are targeted by CSFV.[12–17]

In the early stage of CSF, domestic pigs and wild boars often show acute catarrhal conjunctivitis and shed virus via the lachrymal fluid. Because the nictitating membrane (NM) contains significant amounts of lymphoid tissue and surface as well as glandular epithelium, it might also be involved in the pathogenesis of CSF. The aim of this study was to determine whether the NM might be useful as an additional tissue that is easy to sample for detection of CSFV in experimentally infected wild boars and domestic pigs. Results of FAT, virus isolation, and polymerase chain reaction (PCR) were compared on NM and lymphoid tissue samples for CSF diagnosis.

Because of animal welfare concerns and the need to limit the number of experiments, most of the animals included in this study were part of vaccination-challenge experiments that will not be described here in detail. For challenge infection, the highly virulent CSFV strains ''Koslov''[a] (virus titer $10^{5.5}$ $TCID_{50}$/ml) and ''Alfort 187''[b] ($10^{3.0}$ $TCID_{50}$/ml) and the moderately virulent wild boar–derived CSFV iso-

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Table 1. Detection of CSFV in nictitating membrane (NM) and lymphoid tissues (LYT) of wild boars ($n = 30$) by fluorescent antibody test (FAT), RT-PCR, and cell culture virus isolation (VIR).*

No.	Vaccination	CSFV challenge	Course and time of death (dpi)	Gross pathology	FAT		RT-PCR		VIR	
					NM	LYT	NM	LYT	NM	LYT
1	−	Alfort 187	d (18)	autolysis	−	ND	−	−	−	−
2	−	Alfort 187	d (18)	L,T,C	+	+	+	+	+	+
3	−	Alfort 187	e (16)	L,T,S,C	+	+	+	+	+	+
4	+	Alfort 187	e (27)	−	−	−	−	−	−	−
5	+	Alfort 187	e (27)	−	−	−	−	−	−	−
6	+	Alfort 187	e (27)	−	−	−	−	−	−	−
7	+	Alfort 187	e (27)	−	−	−	−	−	−	−
8	+	Alfort 187	e (27)	−	−	−	−	−	−	−
9	+	Alfort 187	e (27)	−	−	−	−	−	−	−
10	−	Alfort 187	e (27)	L,T,S,C	+	+	+	+	+	+
11	+	11722-WIL	e (28)	L,T	−	ND	−	−	−	−
12	+	11722-WIL	e (28)	L,C	−	ND	−	−	−	−
13	+	11722-WIL	e (28)	L,T,C	−	ND	−	−	−	−
14	+	11722-WIL	e (28)	−	−	ND	−	−	−	−
15	+	11722-WIL	e (28)	−	−	ND	−	−	−	−
16	−	11722-WIL	d (35)	Lu,K	+	+	+	+	+	+
17	+	11722-WIL	e (35)	Lu†	−	−	−	−	−	−
18	+	11722-WIL	e (35)	Lu†	−	ND	−	−	−	−
19	+	11722-WIL	d (43)	L,C,Lu	+	autolytic	+	+	+	+‡
20	+	11722-WIL	d (43)	L,C	−	autolytic	−	−	−	−
21	+	11722-WIL	e (70)	−	−	ND	−	−	−	−
22	+	11722-WIL	e (70)	−	−	ND	−	−	−	−
23	+	11722-WIL	e (70)	Lu†	−	ND	−	−	−	−
24	+	11722-WIL	e (70)	Lu†	−	ND	−	−	−	−
25	+	11722-WIL	e (70)	−	−	ND	−	−	−	−
26	+	11722-WIL	e (70)	G	−	ND	−	−	−	−
27	+	11722-WIL	e (70)	−	−	ND	−	−	−	−
28	+	1236	e (31)	C,Lu,K	+	ND	+	+	+	+
29	+	1236	e (31)	−	+	ND	−	−	−	−
30	+	1236	e (31)	L,C,Lu	+	ND	+	+	+	+

* Course and time of death: d = dead; e = euthanized; dpi = days postchallenge infection; T = tonsil, necrosis with fibrinonecrotic inflammation; L = lymph node, hemorrhage, dendritic hyperplasia; K = kidney, petechiation; Lu = lung, fibrinosuppurative bronchopneumonia and pleuritis; C = colon, fibrinonecrotic inflammation; S = spleen, infarcts; G = stomach, catarrhal stomatitis; ND = not done.

† Lu = lung metastrongylosis.

‡ Virus reisolation positive after several passages.

lates ''11722-WIL''[a] (genotype 2.3 Rostock, $10^{5.5}$ $TCID_{50}$/ml) and ''1236''[a] (genotype 2.3 Uelzen, $10^{3.3}$ $TCID_{50}$/ml) were used. For immunization, the C-strain CSFV was used. The challenge was performed intranasally with cell culture supernatant (11722-WIL, 1236) or whole blood (Koslov, Alfort 187). Most of the infected pigs and wild boars that were not protected by previous vaccination died within 18 days postchallenge infection (PI). The numbers of wild boars and domestic pigs challenged with each strain are shown in Tables 1 and 2, respectively. Reisolation of CSFV from the NM as well as from pooled tonsils and spleen was carried out on PK-15 cells, and CSFV antigen was detected using a mouse anti–CSFV-E2 monoclonal antibody,[c] according to a standardized method.[8]

For RT-PCR, viral RNA was extracted from NM and pooled tonsils and spleen using the ''RNeasy Mini Kit.''[d] The RT-PCR, which has been accredited by the National Reference Laboratory for CSF (Riems Island, Germany), was performed using the following sets of primers: NTR 51 (antisense, 5′-CAACTCCATGTGCCATGTAC-3′, corresponding to nucleotides (nt) 352–371) and NTR 53 (sense, 5′-CATGCCCTTAGTAGGACTAG-3′, nt 88–107) within the 5′ nontranslated region of the CSFV strain Alfort 187. The PCR followed reverse transcription at 42 C/30 minutes and 95 C/3 minutes by 35 cycles of denaturing at 95 C/30 seconds, annealing at 55 C/30 seconds, and extension at 72 C/1 minute. The PCR was ended with a final extension step at 72 C/7 minutes.[7] The amplicons were analyzed by agarose gel electrophoresis and observed by UV transillumination using a gel documentation system. Each RT-PCR assay included 2 negative controls (Diethyl pyrocarbonate[e]–treated water, negative tissue sample) and a specific CSFV-positive tissue sample.

Tissues from NM, tonsils, mandibular lymph node, mesenteric lymph node, and spleen were either snap-frozen in *n*-heptane and stored at −70 C until cryosectioning or immediately fixed in 4% buffered paraformaldehyde and processed for paraffin embedding. For histopathology, sections (3 μm) were deparaffinized and stained with hematoxylin and eosin. Cryostat sections were fixed with acetone (−20

Table 2. Detection of CSFV in nictitating membrane (NM) and lymphoid tissues (LYT) of domestic pigs (n = 9) by fluorescent antibody test (FAT), RT-PCR, and cell culture virus isolation (VIR).*

No.	Vaccination	CSFV challenge	Course and time of death	Gross pathology	FAT		RT-PCR		VIR	
					NM	LYT	NM	LYT	NM	LYT
1	+	Koslov	e (28)†	T,L,K	+	ND	+	+	+	+
2	+	Koslov	e (28)†	T,K	−	ND	+‡	+	+	+
3	+	Koslov	e (28)†	Sk,T,L,Lu,C,K	+	+	+	+	+	+
4	+	Koslov	e (28)†	T,L,C,K,B	+	ND	+	+	+	+
5	+	Koslov	e (28)†	T,C,S,K	+	+	+	+	+	+
6	+	Koslov	e (28)†	T,L,C,K,B	+	ND	+	+	+	+
7	−	Alfort 187	d (9)§	Sk,T,S	+	+	+	+	+	+
8	+	Alfort 187	e (9)§	L,K	−	+	+	+	+	+
9	−	−	e	−	−	−	−	−	−	−

* Course and time of death: d = died; e = euthanized; T = tonsil, necrosis and fibrinonecrotic inflammation; L = lymph node, hemorrhage, dendritic hyperplasia; K = kidney, petechiation; Sk = skin: multifocal to coalescent hemorrhages and necrosis; Lu = lung, bronchopneumonia, parenchymal hemorrhages; C = colon, multifocal fibrinonecrotic inflammation; B = bone metaphyseal growth arrest and osteonecrosis; S = spleen, infarcts; ND = not done.

† Values in parentheses indicate days postcontact infection.

‡ PCR weakly positive.

§ Values in parentheses indicate days postchallenge infection.

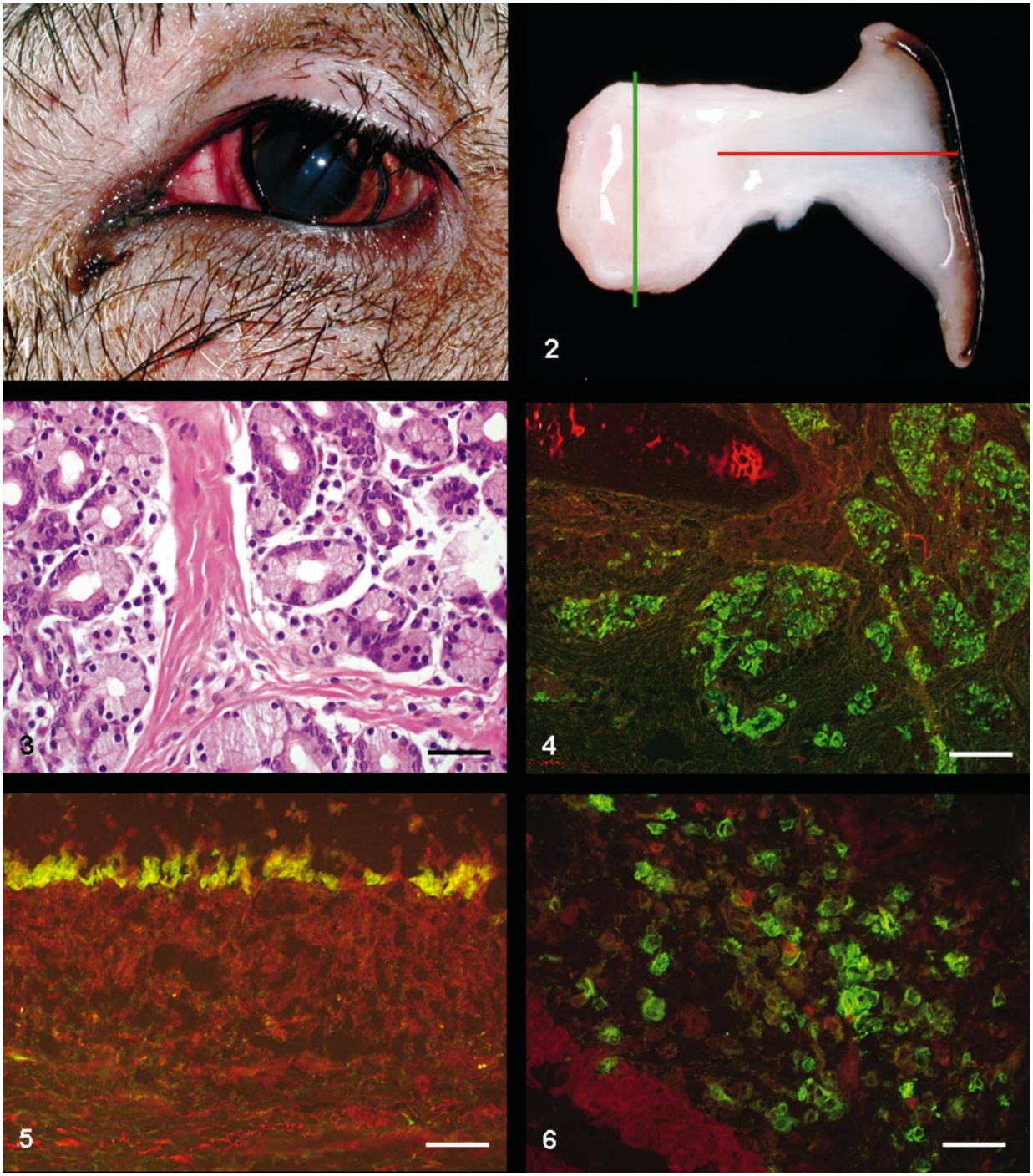
C) for 10 minutes and treated at room temperature with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 20 minutes. Sections were incubated with anti-CSFV monoclonal antibody Bio 275.[f] As secondary antibody, a fluorescein-isothiocyanate–labeled goat anti-mouse IgG2a[g] was used, diluted in 2% BSA–PBS and mixed at the ratio of 3:1 with 0.005% Evans blue[e] solution to reduce background autofluorescence. Sections were sealed in glycerol buffer containing 1,4-diazobicyclo(2,2,2)-octane (DABCO)[e] 25 mg/ml.

At necropsy, characteristic findings for CSF in nonimmunized wild boars included necrotizing tonsillitis, fibrinonecrotic colitis, as well as hemorrhages and edema of the mandibular and mesenteric lymph nodes. More variably present were catarrhal conjunctivitis (Fig. 1), petechiation of kidneys, submucosal hemorrhages of the urinary bladder, pulmonary hemorrhages, splenic infarcts, and catarrhal gastritis. In addition, in domestic pigs, petechiae or multifocal to coalescent cutaneous hemorrhages with dermal infarcts were seen. Wild boars developed consistently fibrinous and hemorrhagic bronchopneumonia, whereas in domestic pigs, metaphyseal growth arrest of the costochondral junction with osteonecrosis was occasionally present. In wild boars and domestic pigs with clinical signs of CSF, the NM showed within areas of nictitating glands hyperemia of submucosal capillaries with slight edema and minimal to moderate perivascular or diffuse infiltration of the interstitium with lymphocytes, plasma cells, and few neutrophils (Figs. 2, 3). Lymphatic follicles of the NM had clusters of medium sized to large cells characterized by hypereosinophilic cytoplasm and karyorrhexis or karyolysis (necrosis). Similarly, surface epithelial cells of the NM in animals with CSF were focally sloughed and showed scattered or diffuse degeneration. Reverse transcriptase–polymerase chain reaction and virus isolation were used to detect viral RNA or CSFV in NM and lymphoid tissue. In 7 of 30 wild boars, there were detectable levels of CSFV RNA in all samples (Table 1). In 8 of 9 domestic pigs, CSFV RNA was detectable in NM and lymphoid tissues (Table 2). However, in 2 animals (Nos. 2 and 8) PCR of NM was only weakly positive.

Immunostaining for CSFV-specific antigen in cryosections of the NM was positive in 3 locations: 1) CSFV antigen was detected in the cytoplasm of a large number of nictitating gland acini (Fig. 4), 2) surface conjunctival epithelium showed brilliant homogeneous cytoplasmic green fluorescence (Fig. 5), and 3) within several lymphatic follicles strong immunostaining was detectable (Fig. 6). The FAT was positive in 7 of 9 NM that were also positive by virus isolation and RT-PCR. Animal No. 29 showed weakly positive immunostaining of the NM, although it was negative for CSFV by the other 2 methods. In the domestic pigs, CSFV-specific immunofluorescence was present in 6 of 8 NM that were also positive by virus isolation and RT-PCR. The NM of domestic pigs No. 2 and 8 were negative by FAT but positive by virus isolation and RT-PCR. In the case of No. 8, a positive FAT signal was present in lymphoid tissues.

To date, use of NM as a diagnostic specimen has been established for the diagnosis of several infectious diseases like scrapie and caprine arthritis encephalitis (Maedi-visna) in sheep or feline infectious peritonitis in felines.[1,5,9] The results presented in this study confirm that immunofluorescence staining of the NM is also useful as an adjunct technique for postmortem diagnosis of CSF. In addition, the ease of collection of the NM makes it a strong candidate for inclusion among routine diagnostic specimens to be collected from wild boars in the field. Furthermore, the NM also appears to be a useful sample for CSF diagnosis in domestic pigs. Nonspecific staining was only present in 1 of 30 NM samples of wild boars. Histopathology did not reveal any lesion that could explain this false-positive result. In general, nonspecific staining is a minor drawback of FAT in a small number of cases that can be resolved by RT-PCR and virus isolation.

The high densities of domestic pig and wild boar populations in central European countries and the predominance of low or moderate virulent CSFV strains in endemic areas



Figures 1–6. **1**, Eye; wild boar No. 3. Acute catarrhal conjunctivitis with mild ocular discharge. **2**, Nictitating membrane; wild boar No. 3. Levels of cryosectioning for fluorescent antibody test are indicated by green (for analysis of nictitating glands) and red line (for surface epithelium and lymphatic follicles). **3**, Nictitating membrane; wild boar No. 3. Slight edema and diffuse moderate infiltration with lymphocytes and plasma cells. Bar = 50 μm. **4**, Nictitating membrane; wild boar No. 3. A large number of glandular epithelial cells show brilliant green cytoplasmic fluorescence specific for CSFV-antigen. Bar = 150 μm. **5**, Nictitating membrane; wild boar No. 3. Cells of the surface epithelium are diffusely positive for CSFV-antigen. In addition, there is yellow autofluorescence because of degeneration and necrosis. Bar = 150 μm. **6**, Nictitating membrane; wild boar No. 3. Within lymphatic follicles, a large number of round to polygonal, medium sized to large cells shows brilliant green cytoplasmic CSFV-specific immunofluorescence. Bar = 75 μm.

because of coevolution lead to mild disease or subclinical infections, which are hard to recognize by veterinarians and hunters. This underscores the need for a more thorough and intense diagnostic effort for CSF in the wild boar population. Because the routine samples (tonsils, kidney, spleen, various lymph nodes, ileum, or bone marrow smear) may give variable results in the FAT,[11] the inclusion of the NM, which shows as strong immunostaining as usually present in CSFV-infected tonsils, could improve the accuracy of CSF diagnosis even in autolyzed carcasses. However, for detection of low-virulent CSFV strains, RT-PCR on blood leukocytes might be the best method.[4]

The NM (third eyelid) of domestic animals is important for the production and distribution of tears, in removing ocular debris and in protection of the eye bulb, and has significant immunologic functions. The observed infection of nictitating gland epithelium makes the NM and its secretions a relevant pathway of viral shedding not only in domestic pigs but also in wild boars. Whether the NM serves as a primary entry site for CSFV during the natural course of infection or whether it is infected during viremia remains to be elucidated. An important advantage of NM as a diagnostic specimen is that it contains all the 3 most relevant cell types for CSFV infection within close anatomical proximity, namely mucosal and gland epithelium and lymphoreticular tissue.

In summary, the data from this study suggest that FAT combined with virus isolation and RT-PCR of the NM is an easily applicable and highly sensitive method for diagnosis of CSF caused by moderate- and high-virulent CSFV strains in wild boar and domestic pigs. However, only the combined use of gross and microscopic pathology, FAT, virus isolation, and RT-PCR methods provide completely reliable results in CSF diagnosis.

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b. European Union Reference Laboratory for CSF, Hannover, Germany.
c. B. Köllner, Riems Island, Germany.
d. QIAGEN GmbH, Hilden, Germany.
e. Sigma–Aldrich, Taufkirchen, Germany.
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