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**Report  
on**

**Annual Meeting of National Swine Fever  
Laboratories**

**Vienna, Austria  
16-17 June 1997**

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## INTRODUCTION

The fourteenth annual EC meeting of the National Swine Fever Laboratories was held in Vienna, Austria on 16-17 June 1997.

The objectives of the meeting were:

- to review the Classical Swine Fever and African Swine Fever situation in Europe;
- to evaluate and discuss results obtained by National Swine Fever Laboratories within the Inter-laboratory comparison test 1997;
- to coordinate standards and methods of diagnosis
- to review research progress and needs for future research concerning the control and eradication of swine fever

The scientific part of the programme was prepared by the Institute of Virology, Hannover Veterinary School, which in accordance with the provision of Council Directive 80/217/EEC acts as the Community Reference Laboratory (CRL) for Classical Swine Fever. The local arrangements were coordinated by Prof. Dr. W. Schuller from the „Bundesforschungsanstalt für Virusseuchenbekämpfung bei Haustieren“, Vienna, Austria.

This report is based on contributions from the representatives from the National Swine Fever Laboratories and the Community Reference Laboratory for Classical Swine Fever. The contributions have been collated and edited by the Community Reference Laboratory for Classical Swine Fever.

## PROGRAMME

### Annual Meeting of National Swine Fever Laboratories Vienna, Austria June 16 - 17, 1997

#### 16 June

**09:00**                    **Opening of the Meeting** by Dr. Jorgen Westergaard, Commission of the European Union

**Session I:**            **The Classical Swine Fever (CSF) and African Swine Fever (ASF) situation in Europe**  
(Chairman: V. Moennig; Rapporteur: P. Have)

**09:30**                    Country reports (30 minutes/country)

**A. African Swine Fever**

Italy/Sardinia (*C. Patta*)

#### COFFEE & TEA

**B. Classical Swine Fever**

Austria (*R. Krassnig*)

Germany (*M. Büttner, V. Kaden*)

Italy (*D. Rutili*)

The Netherlands (*J. van Oirschot*)

Spain (*E. Sur Mora*)

#### LUNCH

**Session II**            **Reports from the Community Reference Laboratory for CSF**  
(Chairman: P. Wilkinson, Rapporteur: M. Hofmann)

**13:30**                    Activities of the Community Reference Laboratory for CSF (*K. Depner*)

**13:50**                    Inter-laboratory comparison test 1997: results and evaluation  
(*K. Depner*)

**14:10**                    The CSF virus database at the CRL (*Irene Greiser-Wilke*)

#### COFFEE & TEA

- Session III**                    **Scientific papers on CSF**  
*(Chairman: S. Edwards, Rapporteur: M. Hofmann)*
- 15:10                    **Genetic heterogeneity of CSFV in Central Europe**  
*(T. Stadejek)*
- 15:30                    **Molecular characterization of CSFV isolates collected in Europe**  
*(I. Greiser-Wilke, M. Hofmann, G.M. De Mia, S. Belak, Z. Pejsak,  
 F. Koenen, D. Paton)*
- 15:40                    **Molecular characterization of CSFV isolates collected in Belgium since 1988**  
*(H. Vanderhallen)*
- 16:00                    **Epidemiology of CSF in Germany from 1993 to 1997** *(J. Fritzemeier)*
- 16:20                    **Automated PCR reading for CSF diagnosis**  
*(A. McGoldrick, P. Lowings, D. Paton)*
- 16:40                    **Biological characterization of CSF isolates from Sardinia** *(G.M. De Mia)*

17 June

- Session IV**                    **Scientific papers on CSF**  
*(Chairman: W. Schuller, Rapporteur: P. Lowings)*
- 09:00                    **CSFV detection in blood from experimentally infected pigs**  
*(M. Hofmann)*
- 09:20                    **Detection of CSFV in muscle samples from experimentally infected pigs**  
*(Barbara Thür)*
- 09:40                    **First results of the surveillance programme for CSF in wild boar in defined areas of Austria** *(Renate Krassnig)*
- 09:50                    **BVDV-investigations: Contact of a cow with a sheep and a pig**  
*(Renate Krassnig)*
- 10:00                    **Pestivirus contamination of fetal bovine serum - still a problem**  
*(M. Büttner)*

COFFEE & TEA

- Session V**                    **Scientific papers on ASF**  
*(Chairman: Castro Portugal, Rapporteur: H. de Smit)*
- 11:00                    Assembly of African swine fever virus *(P. Wilkinson)*
- Session VI**                    **Report on work related to CSF carried out by the sub-groups of Scientific Veterinary Committee**  
*(Chairman: J. Westergaard, Rapporteur: D. Paton)*
- 11:20                    Minimum requirements for NSFL *(S. Edwards)*  
EU Diagnostic manual for CSF *(S. Edwards)*
- 11:40                    Use of marker vaccines during CSF epidemics *(D. Paton, V. Moennig)*
- Session VII**                    **Future work, conclusions and recommendations**  
*(Chairman: D. Rutili, Rapporteur: R. Krassnig)*
- 12:00                    Working programme of the CRL on CSF for 1997/1998  
*(J. Westergaard, K. Depner)*
- 12:15                    Swill feeding legislation *(J. Westergaard)*
- 12:30                    **Conclusions and recommendations**

**Closing of meeting**

## COUNTRY REPORTS - CLASSICAL SWINE FEVER

### The overall situation of CSF in the European Community

The number of outbreaks of CSF reported by Member States is summarized in table 1. The examinations carried out in the European Community for CSF in domestic pigs and wild boar is shown in table 2 and 3. Information on the laboratory capacity for CSF diagnosis within the Community is given in table 4.

Table 1: Number of Outbreaks of Classical Swine Fever Reported by Member States in Domestic Pigs

COUNTRY	1988	1989	1990	1991	1992	1993	1994	1995	1996
Austria	-	19	127	10	23	1	1	1	2
Belgium	2	8	113	0	0	7	48	0	0
Denmark	0	0	0	0	0	0	0	0	0
Finland	0	0	0	0	0	0	0	0	0
France	15	0	4	1	1	1	0	0	0
Germany	3	64*	118**	6	13	100	117	54	4
Greece	0	0	0	0	0	0	0	0	0
Spain	0	0	0	0	0	0	0	0	0
Ireland	0	0	0	0	0	0	0	0	0
Italy	12	11	15	15	20	12	24	42	49
Luxembourg	0	0	0	0	0	0	0	0	0
Netherlands	0	0	2	0	5	0	0	0	0
Portugal	0	0	0	0	0	0	0	0	0
Sweden	0	0	0	0	0	0	0	0	0
United Kingdom	0	0	0	0	0	0	0	0	0
Total	32	83	252	22	39	121	190	97	55

\* Domestic pigs: 31; wild boars: 33

\*\* Domestic pigs: 40; wild boars: 78

\*\*\* Domestic pigs: 38; wild boars: 4

**Number of samples from domestic pig herd investigated for CSF**  
**1996**

Country	Antibody			Virus			Pigs (mil)
	ELISA	NT	ELISA/NT	VI	FAT	ELISA	
Austria	4271	1053		815	398		3,5
Belgium	19291	0	253	1870	1870	865	7,4
Denmark	23625			545		4	11,1
Finland	2643			5	5	8	2
France	78000	17000		54		54	14
Germany	435798						24,28
Greece	824	926	35	10	10	10	
Ireland	1931	0	6	0	0	0	1,6
Italy	14651		23716	296	316	181	9,2
Luxembourg	3463				75		0,072
Netherlands	7000	200	200				15
Portugal	245			1	1		3
Spain	4306	0	38	0	0	0	
Sweden	2672	40	40	0	0	0	3,95
UK	2369	449		0	0	0	6,9
Poland	25223	0	0	28	16	0	16,69

NT: Neutralization test  
Vi: Virus isolation from organs and blood  
FAT: Direct Fluorescent Antibody Test

**Number of samples from wild boar population investigated for CSF**  
**1996**

Country	Antibody			Virus			Wild boars
	ELISA	NT	ELISA/NT	VI	FAT	ELISA	
Austria			178	228	215		>30000
Belgium	0	0	0	0	0	0	10000
Denmark	0			0			?
Finland	0	0	0	0		0	20
France	953	1083		79			450000
Germany				202			600000
Greece							
Ireland	0	0	0	0	0	0	0
Italy	3581		1915	46	21	246(PCR)	?
Luxembourg	9				3		?
Netherlands	0	0	0	0	0	0	3500
Portugal	546			546	546		10000
Spain				0	0	0	?
Sweden	0	0	0	0	0	0	10000
UK	0	0	0	0	0	0	<?
Poland	46	0	0	20	0	1503	72000

NT: Neutralization test

Vi: Virus isolation from organs and blood

FAT: Direct Fluorescent Antibody Test

?: not known

**Weekly capacity of Member States for CSF diagnosis**  
**1996**

Country	National Lab.			Regional Lab.			Pigs (mil)
	Ab	VI	Ag	Ab	VI	Ag	
Austria	2000	500					3,5
Belgium	25000	650	5000				7,4
Denmark	5000	100	500				11,1
Finland	2000	1100	100				2
France	2500	200	900	70000		150	14
Germany	500	100	500	60000	25000	25000	24,28
Greece	950	15	80	450	10	40	?
Ireland	600	30	30				1,6
Italy	2500	200	2000	2500			9,2
Luxembourg	66		2				0,072
Netherlands	60000	3000					15
Portugal	2000	20	20	200		20	3
Spain	?	?	?				?
Sweden	5000	100	100				3,95
UK	3000	3000	3000				6,9

Ab: Antibody examination (Neutralization test, ELISA)

Vi: Virus isolation from organs and blood

Ag: Antigen detection (FAT, ELISA)

?: not known

## **Austria**

*R. Krassnig*

**R. Krassnig** reported that no outbreaks of CSF had occurred in domestic pigs in '96 and '97. However, some seropositive wild boar had been found in Gensendorf near the border to the Czech Republic.

*Moennig* asked for an update on wild boar and plans of eradication.

*Krassnig* answered that some outbreaks had occurred in Lower Austria during 1990-1993 and that wild boar were investigated for virus and antibodies. In the area of Gensendorf farms were investigated and a surveillance was carried out in wild boar including increased hunting.

*Weber* added that an eradication programme was in place including shooting out of animals. So far no virus-positive animals had been detected this year.

*Moennig* asked whether age of wild boar was recorded. In some cases it was and it showed that all age groups were represented.

*Wilkinson* asked whether the wild boar were located in a discrete area or whether there was contact with populations in other countries.

*Krassnig* replied that crossing the border to the Czech Republic could occur, however some natural borders would tend to limit such movements.

## **Germany**

*M. Büttner, V. Kaden*

### Domestic pigs

Having recorded only 4 outbreaks in domestic pigs in 1996 the situation became worse in 1997 with 43 outbreaks occurring until now in several Länder. 202 wild boar cases were found in 1996 and 62 in 1997. There had been changes in diagnostic procedures, adding PCR and sequencing on a routine basis. Strains were still typed using a panel of monoclonal antibodies (mAbs).

*van Oirschot* asked for possible causes for outbreaks.

*Büttner*: It appeared that swill feeding, including meat from infected wild boar and illegally imported meat, was important in initiating outbreaks whereas secondary outbreaks were often caused by neighbourhood contact and animal movements.

*Bjönerod* asked whether the situation could be considered under control.

*Büttner* replied that diagnosis is safe and prompt but that in the present situation no area can be considered entirely safe in terms of potential new outbreaks.

### Wild boar

The wild boar population was free from CSF in the beginning of the '80ies and from '88 an increasing number of CSF cases occurred in wild boar. *Dr. Kaden* summarized the experiments of oral vaccination and concluded that in Lower Saxony it appeared to have been successful in eradicating disease. The vaccine used contains 500.000 PD50/bait and is highly stable.

*Rutili* asked about hunting seasons and it was told that wild boar are open for hunt all of the year (except pregnant and nursing sows).

*Hofman* asked whether vaccine virus was sometimes isolated from wild boar.

*Kaden* said that all isolated were typed and that they were all field viruses.

*Albina* noted that seropositive wild boar were still found in the North-Eastern part of France and asked about the situation in adjacent areas of Germany. Also he would be interested in testing oral vaccination in that particular area of France.

## Italy

*D. Rutili*

Classical swine fever (CSF) is still endemic in Sardinia where 43 outbreaks in pig farms occurred during 1996 whereas 2 cases of disease have been reported in wild boar. Starting from 1997 the eradication plan for CSF has been joined to African Swine Fever programmes implemented in Sardinia. In mainland Italy 3 outbreaks in domestic pigs and one case in wild boar have been reported.

All the herds involved are of little size or *self consumption* type and the 1465 pigs affected were slaughtered. The source of the infection, in most outbreaks, is very likely swill feeding. In Sardinia the contact of free ranging pigs with wild boar is also a frequent source of infection, mainly in the province of Nuoro. Molecular studies do not show any relationship among the last Sardinian isolates and the continental ones.

In 1996 has been completed the serological monitoring of national pig population against CSF. The herd sampling was based upon a 95% probability of finding seropositive animals if 10% of animals in the herd were positive. A random sampling of pigs in different units of the herds was used. The survey involved 6,425 farms and 152,278 animals were tested. No evidence of infection was found in all the regions controlled.

In spite of these results, during the first six months of 1997, 3 outbreaks of CSF have been reported. Infected animals imported from a EU country and swill feeding have been detected as the source of the infection.

On the basis of the epidemiological trend of CSF in Italy, the following statements can be drawn:

- CSF is still present in two risk areas of Sardinia where the wild boar, the practices of farmers and hunters and free ranging pigs diminish the efforts of the Veterinary Service to ~~eradicate the disease~~.
- The contaminated meat is acquiring an important role in spreading the CSF virus threatening the eradication programmes due to the lack of a practical and effective control system because it seems that the controls at origin are unreliable.
- The serological surveillance programmes give a moderate contribution to the control and eradication of CSF in the most part of the Italian regions.
- The little size pig or wild boar rearing units linked to touristic enterprises are consistently increasing in many regions of the country and together with increased trade of meat make difficult to prevent the introduction of CSF virus in free areas.

### **The Netherlands**

*A. Stegeman, A. Elbers, A. de Smit, C. Terpstra, R. Moormann, J. van Oirschot  
Institute for Animal Science and Health (ID-DLO), Lelystad, The Netherlands  
Animal Health Service, Deventer, The Netherlands*

Approximately 270 cases of classical swine fever (CSF) have been diagnosed from February 4 to June 14, 1997. The outbreak is confined to the pig dense region in the south-eastern part of the Netherlands. Epidemiological investigations suggest that a transport vehicle harbouring the virus was the most likely cause of introduction. This was supported by the finding that sequences of the isolate of the first case were found almost identical to the Paderborn virus isolated in Germany in January 1997. At the time the first case was diagnosed, it was estimated that 30-40 other herds were already infected, mainly due to transport of pigs. After the installment of standstill of livestock and other zoosanitary measures "neighbourhood infection" is considered the most likely mode of virus transmission. Epidemiological investigations also suggest that sows became infected by artificial insemination with semen from infected boars.

### **DISCUSSION**

*Westergaard* informed that until now almost 2 mill. animals had been killed as a consequence of stamping out, preventive slaughtering and market support.

*Wilkinson* asked why a 10 km protection zone was established.

*de Smit* said that it was due to an overreaction and that it probably was a mistake, leading to more than 20.000 clinical inspections over a very short period of time and exhausting veterinary and logistic resources unnecessarily.

*Paton* asked about virulence of the strain.

*de Smit* said that it was typical of CSF, i.e. no single typical picture but rather a large variation from no signs to severe acute cases.

*Pejsak* noted that CSF in densely populated regions constitutes a major risk to Europe and asked whether any structural changes were foreseen. The answer was no.

The laboratory in Lelystad has until now carried out 150.000 virus isolations and some 300.000 CTB tests in the course of the epidemic.

### **Spain**

*E. Sur Mora*

45 outbreaks in 9 municipalities in the province of Lerida has so far been recorded following introduction of infected pigs from NL. Dr. de Smit asked how many outbreaks were directly linked to import of pigs from NL. This was not known.

## COUNTRY REPORTS - AFRICAN SWINE FEVER

### Italy/Sardinia

#### PLAN FOR ERADICATION OF ASF FROM SARDINIA

*Patta C., Oggiano A., Cattina A., Madrau P., Sarria A., Sanna M.L.*

African swine fever (ASF) is still present in Sardinia even if confined in the Province of Nuoro. In the rest of the island neither outbreaks nor seropositive pigs have been reported in this year. In the January of the 1997, 2 seropositive pigs have been found in a holding located in the Commune of S.Vito, Local Health Unit n°8 (Cagliari) District of Muravera. The owner of the seropositive pigs was native of Arzana (Nuoro) and an illegal movement of pigs represented the source of this finding; all the animals have been killed and destroyed. The controls carried out in this area have shown no other traces of the infection.

In the "endemic area" of the Province of Nuoro ( Districts of Nuoro, Siniscola, Sorgono and Lanusei), 65 outbreaks occurred, 30 of them (46%) in the eight Communes ( Orgosolo, Desulo, Villagrande Strisaili, Arzana, Baunei, Triei, Urzulei, Talana) of the "high risk area". Outside the "high risk area", 35 outbreaks occurred in 15 Communes probably linked to illegal movement of animals and pig meats; nevertheless the situation is largely improved when compared with the high number of outbreaks (145) recognized in 1995. A further decrease in the number of outbreaks has been registered until the end of May 1997 (only 9 outbreaks).

Serological controls on pig holdings have been continued all over the island. In particular in the Province of Nuoro 5,866 pig holdings have been checked (Tab. 1), and in the rest of the island the control activities have involved 8,995 holdings.

From the second semester of 1995 up to December 1996 a declining trend of seropositive farms has been constantly observed both in the high risk area (4.7% out of 12.9%) and in the rest of the endemic area (0.1% out of 0.2%).

This favourable situation is presumably due to the following:

- 1) the intense stamping out operations performed around the outbreaks, above all when the outbreaks occurred in areas where a high number of free ranging pigs are kept.
- 2) In 6 out of 8 Communes of the high risk area, the municipality have implemented rules in order to time limit the use of communal land and the farmers were forced to enclose animals in the summer period (from 30 of June to 30 of September); this fact could have limited the contact between groups of animals which is often related to the spread of the disease.

Even if the results seems to confirm the validity of the strategy, it must be underlined that in the Commune like Orgosolo and Desulo the farmers cooperation is still not sufficient. The support of The Police was request in several occasions in order to solve this social situation that represent a real threat for the eradication program.

The high percentage of outbreaks of ASF reported in several Communes of the endemic area has been considered with great concern, for this reason and in agreement with the suggestion of European experts it has been decided to change the control strategy defining the risk at communal level.

In order to estimate the risk of having outbreaks in commune we have considered the followings parameters:

- 1) the zootechnical situation: the presence of the communal land where the outdoor husbandry system was practised;
- 2) the epidemiological situation: n° of outbreaks and percentage of seropositive farms detected during the eradication plans activity;
- 3) the territorial continuity with communes having outbreaks;

We have considered two different level of risk:

**High risk:** all the communes (n° 23) where we have all the above parameters at the same time.

**Low risk:** all the communes (n° 32) where these parameters are partially present, if a low number of outbreaks were recognized, they were easy eradication, furthermore the breeding system and the border with high risk communes seemed to conduce stronger measures.

The rest of the Province of Nuoro is included in the surveillance area.

This evaluation is more restrictive than in the past, these findings was preliminary to the new eradication strategy that has allowed, in the 1997, to concentrate all the vets in the same Province in order to retest all the holdings. At the end of May all the holdings in the Province of Nuoro have been checked. This strong intervention, we hope, could allow a control level of the disease close to the eradication.

In the rest of Sardinia the eradication program will be performed by the Local Health Units vets.

#### Result of the serological investigations on wild boar

Two cases of ASF were reported in wild boar found dead in the Communes of Nuoro and Orgosolo. Table 2 shows the results of the serological tests carried out onto wild boar shot during the hunting season November 1996 – January 1997 in the endemica area. From the last hunting season up to now a declining trend of seropositive wild boar has been observed both in the high risk area ( 11.3% out of 17.5%) and in the rest of the endemic area (1.7% out of 2.7%). In the Communes of Irgoli Loculi and Onifai the seroprevalence has fallen at 3% in comparison with 9.4% in the 1995.

TABLE 1: OVERALL RESULTS OF THE SEROLOGICAL TESTS CARRIED OUT IN 1996 AND NUMBER AND LOCATION OF OUTBREAKS IN THE PROVINCE OF NUORO

DISTRICTS	HOLDINGS TESTED	HOLDINGS POSITIVE	SAMPLES TESTED	PIGS POSITIVE	OUTBREAKS
MACOMER	464		3006		
NUORO	1231	20*	11703	95*	33**
SINISCOLA	1144	8*	8784	17*	3
SORGONO	596	17*	5885	97*	10
ISILI	615		3428		
LANUSEI	1816	53*	26256	335*	19
<b>Tot Prov. NU</b>	<b>5866</b>	<b>98*</b>	<b>59062</b>	<b>544*</b>	<b>65</b>

\* These numbers also includes holdings and pigs found seropositive during ASF outbreaks

\*\* Including 2 cases in wild boar

TABLE 2: RESULTS OF THE SEROLOGICAL TESTS ON TO WILD BOAR SHOT IN THE ENDEMIC AREA

	HUNTING SEASON DEC 93 – JAN 94			HUNTING SEASON DEC 94 – JAN 95			HUNTING SEASON DEC 95 – JAN 96			HUNTING SEASON DEC 96 – JAN 97		
	Tested	Pos	%	Tested	Pos	%	Tested	Pos	%	Tested	Pos	%
<b>HIGH RISK AREA (8COMMUNES)</b>	179	29	16.2	261	74	28.4	303	53	17.5	381	43	11.3
<b>IRGOLI, LOCULI AND ONIFAI</b>	66	17	25.8	158	34	21.5	127	12	9.4	167	5	3
<b>REST OF THE ENDEMIC AREA</b>	746	29	3.9	1167	69	5.9	740	20	2.7	809	14	1.7
<b>TOTAL</b>	<b>991</b>	<b>75</b>	<b>7.6</b>	<b>1586</b>	<b>177</b>	<b>11.2</b>	<b>1170</b>	<b>85</b>	<b>7.3</b>	<b>1357</b>	<b>62</b>	<b>4.6</b>

## DISCUSSION

### *C. Patta*

In 1997 only 9 outbreaks had been recorded compared to 65 in 1996. These were all located in the hyperendemic area of the province of Nuoro. Also, a declining seroprevalence has been observed. The improved situation is mainly due to reduced use of communal land and enclosure of animals during summer months. In wild boar a decreased seroprevalence has also been observed.

*Wilkinson* noted that it was encouraging to see such a small number of ASF outbreaks and asked whether controls and checks could be implemented in the whole area. Drs. Patta and Rutili responded that the efforts of the veterinary service had reached its utmost and that further actions were dependant on help from the police.

*Moennig* asked whether restrictions could be properly implemented on farms having access to privately owned land. Dr. Patta responded that this was not a problem.

## **REPORTS FROM THE COMMUNITY REFERENCE LABORATORY FOR CSF**

### **Activities of the Community Reference Laboratory for CSF**

*K. Depner*

#### **I. Contractual duties.**

The functions and duties are specified in Annex VI of Council Directive 80/217/EEC (Official Journal of the European Communities No L 166 of 8.7.1993).

#### **II. Objectives for the period October 1996 - September 1997.**

1. Collecting and editing of material for a report covering the annual meeting of National Swine Fever Laboratories held at Alghero, Sardinia. The meeting covers sessions related to CSF and ASF.
2. Collection and editing of material for a report covering the workshop on diagnostic procedures and measures to control CSF in domestic pigs and the European wild boar held at Pulawy, Poland.
3. Selection and inoculation of pigs for the production of CSF sera for the inter-laboratory comparison test to be carried out by the National Laboratories of Member States.
4. Quality control and distribution of the reagents prepared to carry out the inter-laboratory comparison test.
5. Analysis of results submitted by Member States as regards the inter-laboratory comparison test.
6. Review of the proposed standardized methods for the virus neutralization tests for CSF and BVD.
7. Prepare programme and working documents for Annual Meeting of National Swine Fever Laboratories, 1997.
8. Re-stocking of CSF video and preparation of video in Swedish and Finish.
9. Conduct CSF workshop on CSF pathology, diagnostic procedures, epidemiology and disease control. (Duration: 5 days - max. 12 participants).
10. Perform check on stock of cell cultures, strains of virus and selected reagents.
11. Examine new isolates of CSF virus submitted by NSFL from Member States and build up virus collection.
12. Pathogenicity studies of the chronic form of CSF in domestic pigs infected with CSF virus isolates from Lower Saxony.

13. Evaluation of data from Member States on molecular epidemiology of CSF for the purpose of the CRL.
14. Support on ongoing epidemiological research projects in Belgium, The Netherlands and Germany on recent outbreaks of CSF.

#### DISCUSSION

*Alenius*: Way of inoculation for animal experiments? *Depner*: Intranasally.

*de Smit*: Were pigs killed or allowed to die?

*Depner*: 2 died at night, 1 euthanized.

*Alenius*: Were BVDV2 strains tested in pigs?

*Depner*: No, not yet.

*de Smit*: Adds, that they had done such experiments: no clinical symptoms, after challenge with Brescia some pigs showed no disease signs, some died, some were ill but recovered.

*Westergaard*: Suggested to drop ASF comparative testing from Hannover's duties, and to ask another lab instead if prepared to conduct.

#### **Inter-laboratory comparison test 1997: results and evaluation**

*K.R. Depner*

The 1997 inter-laboratory comparison test included 33 European laboratories from 30 countries. Apart from the member states several non-member states from Eastern and Central Europe were invited to participate. The laboratories were supplied in November 1996 with a set of five lyophilised porcine sera. The sera had to be tested for the presence of antibodies against CSF and BVDV. All sera were produced in animal experiments conducted at the CRL.

The distribution of sera to and collection of results from Bulgaria, Byelorussia, Czech Republic, Estonia, Hungary, Lithuania, Latvia, Poland, Romania, Russia and Ukraine was coordinated by the National Veterinary Research Institute, Pulawy, Poland. The evaluation of the results from the Eastern and Central European countries will take place on a Seminar on CSF at the Estonian National Veterinary Laboratory, Tallin, Estonia during 23-23 October 1997.

The laboratories of Member States received additionally to the sera 2 ml of lyophilized Classical Swine Fever Virus (CSFV) for the determination of the virus titre. The introduction of CSFV with an unknown titre to the *Inter-laboratory Comparison Test 1997* was proposed at the last National Swine Fever Laboratory (NSFL) meeting in Alghero/Sardinia.

#### Origin of test sera

##### **Serum A (F4) - Wild boar serum sample with low antibody titre for CSF**

A pregnant wild boar was inoculated intranasally with CSFV isolate Diepholz 1/Han94 at about 87 to 92 days of gestation. The wild boar did not show any signs of illness post infection (p.i.) but seroconversion was noticed. Twenty eight days p.i. birth was given to six clinically healthy offsprings. One of the newborn proved to be viraemic until death when 39 days of age. Except for poor growth no other symptoms were noticed in this piglet. The non-viraemic litter mates remained healthy, although they had close contact to the persistently infected piglet. Shortly after birth high titres of neutralizing antibodies against CSFV were measured in the serum samples of these offsprings. A continual decrease of the titre occurred during the following months. The serum used in this years' *Inter-laboratory Comparison Test* was

collected 12 months after birth from one of the healthy wild boar piglets. The antibodies in the serum are most probably of maternal origin.

**Serum B (475/22)** - Serum sample with low antibody titre for CSF

The serum was obtained from one of ten weaner pigs inoculated intranasally with 250 TCID<sub>50</sub> of the CSFV isolate Diepholz 1/Han94. The isolate was derived from a diseased pig out of a CSF outbreak in Lower Saxony in February 1994. The isolate was characterized as highly virulent because 90% of intranasally inoculated weaners died of acute symptoms typical of CSF (see DEPNER et al., Wien. Tierärztl. Mschr. 81 (1994) 370-373). After an incubation period of seven days this pig became febrile and showed a strong clinical reaction typical for CSF. The serum was collected on day 22 p.i. when the animal had to be euthanized while moribund.

**Serum C (540/51)** - Serum sample with high antibody titre for CSF

The serum was obtained from a weaner pig inoculated intranasally with the CSFV isolate Visbek/Han95. The isolate was derived from a diseased pig out of a CSF outbreak in Lower Saxony in 1995. The serum was collected on day 51 p.i. No clinical symptoms typical for the acute form of CSF were noticed in this animal.

**Serum D** - Serum with no antibodies against pestiviruses

**Serum E (24/33)** - Serum sample with high antibody titre for CSF

The serum was obtained from a weaner pig inoculated intranasally with the CSFV isolate Diepholz 1/Han94. The serum was collected on day 33 p.i. No clinical symptoms typical for the acute form of CSF were noticed in this animal.

### Neutralization titre of sera used in the Inter-laboratory comparison test 1997

Virus	A	B	C	D	E
Alfort/187	0.7	1.7	2.6	<0.7	1.7
Diepholz	<u>0.8</u>	<u>0.8</u>	>2.9	<0.7	<u>1.7</u>
Visbek	0.8	1.4	<u>3.1</u>	<0.7	2.3
487/93/wb	1.1	1.4	3.2	0.7	1.9
NADL	<0.7	<0.7	1.4	<0.7	0.8

A (F4): wild boar, 12 months, maternal antibodies

B (475/22-V94/1): weaner, 22 dpi, died due to CSF

C (540/51-96/1a): weaner, 51 dpi, recovered from CSF

E (24/33-V95/3): weaner, 33 dpi, recovered from CSF

Inter-laboratory Comparison Test 1997 - Code of sera

Country	F/4				Serum E
	Serum A	Serum B	Serum C	Serum D	
Austria (1)	55	125	153	30	101
Belgium (2)	2	56	115	86	59
Denmark (3)	47	122	155	15	22
Finland (4)	85	36	161	69	7
France/Alfort(5)	92	131	63	104	126
France/Ploufragan (6)	46	128	703	138	90
Germany (7)	82	139	27	113	11
Great Britain (8)	136	52	124	14	23
Greece/Athens (9)	21	162	95	149	116
Greece/Thess. (10)	171	13	93	29	76
Irish Rep. (11)	9	28	66	170	24
Italy (12)	151	75	169	143	49
Luxembourg (13)	83	39	127	53	111
Netherlands (14)	109	154	1	150	48
Portugal (15)	91	54	130	40	103
Spain (16)	114	144	41	174	133
Sweden (17)	64	132	175	45	81
CRL (18)	264	232	275	245	281
Norway (26)	214	244	241	274	233
Poland (27)	371	313	393	326	424
Switzerland (32)	409	429	466	470	411

## Results obtained in the neutralization tests for CSF (1997)

Lab code	Neutralization titre (ND <sub>50</sub> )					BT	Test	Test virus	cell culture
	A	B	C	D	E				
1	0,8	2,5	3,0	<0,3	2,1	100	NPLA	Alfort/187	PK(15)
1	0,7	1,9	2,8	<0,3	1,8	100	NIFT	Alfort/187	PK(15)
2	0,7	1,5	2,3	<0,7	1,4	106	NPLA	Alfort/187	PK(15)
3	0,8	1,6	>2,4	<0,3	2,0	200	NPLA	Alfort	PK(15)
4	<u>&lt;0,7</u>	1,3	3,1	<0,7	1,6	100	NPLA	Alfort/187	PK(15)
5	<u>&lt;1,0</u>	<u>0,5</u>	2,2	<1,0	<u>1,1</u>		NIFT	Alfort/A19	PK(15)
6	<u>&lt;0,7</u>	<u>0,8</u>	2,0	<0,7	<u>1,0</u>	100	NIFT	Alfort/A19	PK(15)
6	<u>&lt;0,7</u>	<u>1,0</u>	2,0	<0,7	<u>1,1</u>	100	NPLA	Alfort/187	PK(15)
7	0,9	1,6	2,8	<0,3	1,6	30	NPLA	Alfort	STE
8	<u>&lt;0,7</u>	1,4	2,6	<0,7	1,6	56	NPLA	Alfort/187	PK(15)
9	<u>&lt;0,7</u>	1,0	2,4	<0,7	1,5	100	NIFT	Alfort/187	PK(15)
10	<u>&lt;0,7</u>	1,6	2,4	<0,7	1,5	100	NIFT	Alfort/187	PK(15)
11	0,6	1,6	2,8	<0,3	1,8	126	NPLA	Alfort/187	PK(15)
12	<u>&lt;1,0</u>	1,6	2,6	<1,0	1,6	100	NIFT	Alfort/187	PK(15)
14	<u>&lt;1,1</u>	2,1	3,1	<1,1	2,0	256	NPLA	Brescia	PK(15)
16	0,7	1,3	2,8	<0,7	1,6	199	NPLA	Alfort/187	PK(15)
17	<u>&lt;0,7</u>	1,3	2,5	<0,7	1,6	100	NPLA	Alfort/187	PK(15)
18	0,7	1,7	2,6	<0,7	1,7	256	NPLA	Alfort/187	PK(15)
18	0,8	<u>0,8</u>	2,9	<0,7	1,7	320	NPLA	Diepholz	PK(15)
18	0,8	1,4	3,1	<0,5	2,3	256	NPLA	Visbek	PK(15)
18	1,1	1,4	3,2	<u>0,7</u>	1,9	192	NPLA	WB487/93	PK(15)
27	<u>&lt;0,7</u>	<u>1,0</u>	2,0	<0,7	1,2	178	NPLA	Alfort/187	SK-6
32	<u>&lt;0,4</u>	2,1	3,3	<0,4	2,1	<b>13</b>	NIFT	Alfort/187	SK-6

## Results obtained in the neutralization tests for BVD and BD (1997)

Lab code	Neutralization titre (ND <sub>50</sub> )					BT	Test	Test virus	cell culture
	A	B	C	D	E				
2	<0,3	<0,3	1	<0,3	0,7	32	CPE	NADL	BT
3	<0,3	0,3	1	<0,3	0,6	51	CPE	SVS259	MDBK
4	<0,7	0,7	1,5	<0,7	0,7	100	CPE	NADL	BT
5	<0,3	0,9	1,5	<0,3	0,9		CPE	NADL	NBL1
7	<0,3	1	1,4	<0,3	<0,3	100	CPE	Grub	MDBK
8	<0,7	<0,7	0,7	<0,7	0,7	56	CPE	Oregon	BT
9	<0,7	<0,7	1,7	<0,7	0,7	100	CPE	NADL	MDBK
10	<0,7	0,7	1,6	<0,7	0,8	100	CPE	NADL	MDBK
11	<0,3	0,9	1,7	<0,3	1,2	83	CPE	NADL	FCK
14	<1,1	<1,1	<1,1	<1,1	1,2		NPLA	Oregon	EBTR
14	<1,1	<1,1	1,1	<1,1	1,1	39	NPLA	v.EE	EBTR
16	<0,3	0,3	1,3	<0,3	0,8	100	CPE	NADL	MDBK
17	<0,7	<0,7	1,3	<0,7	0,7	100	NPLA	NADL	BT
18	<0,7	<0,7	1,4	<0,7	0,8	100	NPLA	NADL	FCK
26	<0,3	<0,3	0,9	0,3	0,6	100	CPE	NADL	BT
27	<u>1,2</u>	0,3	0,9	<u>1,5</u>	1,2	100	CPE	NADL	MDBK
32	<0,4	1,4	<u>2,1</u>	<0,4	<u>1,6</u>	63	CPE	NADL	FCK
6	<0,7	<0,7	1,3	<0,7	0,7	100	NPLA	Aveyron	PK(15)
6	<0,7	0,8	1,3	<0,7	1	100	NIFT	Aveyron	PK(15)
14	<1,1	<1,1	1,8	<1,1	1,1	63	NPLA	Strain F	EBTR

### Detection of CSF antibodies with different ELISA (1997)

Lab code	ELISA	Serum sample				
		A	B	C	D	E
1	CTB	<u>neg</u>	pos	pos	neg	pos
2	CTB	<u>neg</u>	pos (8*)	pos (6)	neg	pos (1)
3	Block	<u>neg</u>	<u>neg</u>	pos (35)	neg	<u>neg</u>
4	CTB	<u>neg</u>	pos	pos	neg	pos
5	SANOFI	<u>neg</u>	neg	pos	neg	pos
6	in house	?	pos	pos	neg	pos
6	CHEKIT	pos	?	pos	neg	pos
6	SANOFI	pos	pos	pos	neg	pos
7	CHEKIT	?	?	pos	neg	pos
8	CTB/UK	<u>neg</u>	<u>neg</u>	pos	neg	<u>neg</u>
9	CTB	<u>neg</u>	pos	pos	neg	pos
11	CTB	<u>neg</u>	pos (1)	pos (20)	neg	pos (2)
12	CTB	<u>neg</u>	pos	pos	neg	pos
13	CTB	<u>neg</u>	?	pos	neg	pos
13	Ceditest	<u>neg</u>	?	pos	neg	pos
13	CHEKIT	<u>neg</u>	<u>neg</u>	pos	neg	pos
14	Ceditest	<u>neg</u>	pos	pos	neg	pos
17	CTB	<u>neg</u>	pos (2)	pos (16)	neg	pos (2)
26	CTB	<u>neg</u>	pos	pos	neg	pos
27	CTB	<u>neg</u>	?	pos	neg	pos
32	Block/VI	<u>neg</u>	<u>neg</u>	pos (32)	neg	?
32	Indirect/VI	<u>neg</u>	pos (16)	pos (32)	neg	pos (16)
32	CTB	<u>neg</u>	<u>neg</u>	pos (8)	neg	pos (1)

\*: IU - International Unit ? : questionable

### Titration of CSF virus (1997)

Lab code	Log <sub>10</sub> TCID <sub>50</sub> /ml		cell culture
	I	II	
1	1,9*	2,2	PK(15)
2	2,0	2,0	PK(15)
3	4,4	4,8	PK(15)
4	4,0	5,3	PK(15)
4	4,5	5,0	STE
5	4,2	4,2	PK(15)
6	5,3	5,3	PK(15)
8	3,8	3,8	PK(15)
9	3,0	3,0	PK(15)
11	4,2	4,1	PK(15)
12	3,7	4,5	PK(15)
14	4,9	6,0	PK(15)
14	4,8	5,8	PK(15)
17	3,0	3,0	PK(15)
18	5,5	5,5	PK(15)
32	2,0	2,0	

\*: 10<sup>1,9</sup> TCID<sub>50</sub>/ml

### DISCUSSION

*de Smit*: Were some of the differences caused by several Alfort strains used in the VNT?

*Depner*: Only information provided by the laboratories was used.

*Edwards*: Virus titrations should be confirmed with low titer stocks.

*Wilkinson*: How can these differences in various labs be explained?

*Edwards*: Maybe due to storage conditions/duration of shipment of virus. Therefore it is suggested to send one ampule back to CRL to test for loss of titer due to transport.

*Hofmann*: Was a low solubility of the lyophilized virus also observed in other labs?

*Koenen*: Yes, also Ag ELISA with this sample was negativ.

*Depner*: Virus was only provided for titration, neutralization test should be done with own virus.

*Edwards*: Emphasizes importance of a common protocol.

## The CSF virus database at the CRL - an update

*Irene Greiser-Wilke*<sup>1</sup>, *Klaus Depner*<sup>2</sup>, *Joerg Fritzemeier*<sup>1</sup>, *Volker Moennig*<sup>2</sup>  
*Veterinary School Hannover, Institute of Virology*<sup>1</sup> and *Community Reference Laboratory for CSF*<sup>2</sup>, *Bünteweg 17, 30559 HANNOVER*

A database containing all currently available information concerning the CSF virus strains and isolates kept in the CRL / Institute of Virology in Hannover has been established and is being continuously updated. The objectives of the CSF virus collection and database are the following:

- To keep stocks of CSF viruses from all outbreaks in or near the EU, and - if available - from all over the world. In order allow the collection to become as complete as possible, the Institute of Virology has an unlimited import permit, so that new isolates can be sent to us without delay. *A copy of the import permit must be included in every shipment mailed to the CRL.*
  - To make CSF virus strains and isolates available for genetic and antigenic characterisation, and for epidemiological and pathogenic studies.
  - To collect and pool all available data.
- Unless otherwise stated, the CSF viruses are available to authorized swine fever laboratories from the CRL upon request.

### Running projects

The database has been ported from PARADOX 3.0 (BORLAND) for DOS to PARADOX 4.5 for MS Windows 3.11/xx.

The collection of isolates has been increased from 150 in the last reporting period to about 400. Epidemiological data (place and year of isolation, isolation from domestic pig or wild boar) were recorded, including the EU-outbreak numbers (if available). The collection includes isolates from Germany from former and from the 1997 outbreaks, from Austria, Poland, Hungary, Tschechia, Belgium, Estonia, Hungary, The Netherlands, as well as from Malaysia, Brazil, Jpan and USA.

Rapid identification of new CSF isolates can be performed by genetic typing. As part of the cooperative CSF Fair project 3-CT95-0707, sequence databases including fragments of the 5' nontranslated region (5'-NTR), the E2- and the NS5B-genes are being constructed. In addition, a computer program, the HLA-*SequiTyper* (PHARMACIA BIOTECH) has been adapted for evaluation of new sequences and can now be used for rapid typing of new isolates.

As the main server in the Veterinary School Hannover does not allow to give passwords and cannot implement a search engine for the database, a new server has been installed at the CRL, and the home page will be accessible within 1997. Until that time, the virus database can only be supplied as a hard copy upon request.

### DISCUSSION

*Belak*: Other pestiviruses other than CSFV collected at CRL?

*Greiser-Wilke*: Collected yes (many BVDV), not yet sequenced.

*Wilkinson*: In what form should viruses be sent?

*Depner*: As cell culture supernatants, are less tedious to grow.

*Wilkinson*: Any sequence differences observed between organ material and cell culture supernatant?

*Wilke*: No, sequence seems to be more stable than commonly thought.

## Preparation of international standard sera for classical swine fever

*Steven Edwards<sup>1</sup> and Klaus Depner<sup>2</sup>*

<sup>1</sup>*OIE Reference Laboratory for Classical Swine Fever, Weybridge, UK*

<sup>2</sup>*OIE and EU Reference Laboratory for Classical Swine Fever, Hannover, Germany*

The Office International des Epizooties (OIE) has requested the two reference laboratories in Weybridge and Hannover to collaborate on the preparation of international reference sera for use in the standardisation of serological tests for classical swine fever (CSF). A number of other national and international reference laboratories were asked to participate in the evaluation process (Pulawy, Poland; Nepean, Canada; Ames, USA; Geelong, Australia; Tokyo, Japan).

### Methods

The „OIE Guidelines for International Reference Standards for Antibody Assays“ have been followed. These include the preparation of positive, weak positive, and negative candidate sera. The steps taken were as follows:

- I. Preparation of a negative serum - taken in bulk from a pig in a CSF-free country (UK).
- II. Screening of candidate positive sera from the Hannover reference laboratory.
  - A. several sera were rejected due to cross reactivity with ruminant pestiviruses (especially border disease virus)
  - B. a serum was selected from a pig recovered from experimental infection with CSF strain Alfort/187
- III. Preparation of trial dilutions
  - A. positive serum was mixed with negative serum to achieve suitable activity levels corresponding with the OIE criteria
  - B. serum mixtures were gamma-irradiated (25-30 kilogray) and freeze dried
- IV. Sets of identical aliquots were distributed to the participants in the evaluation.
  - A. difficulties were encountered with importation regulations to certain countries
  - B. not all results are yet complete, however it is already apparent that further candidate serum mixtures need to be prepared and a further distribution carried **out**

## Results

For the tabulation, all results for neutralisation test (NPLA) have been converted to the equivalent of reciprocal initial dilutions.

### Dilution of positive to negative serum in the samples

Lab	1/5	1/10	1/25	1/50	1/100	1/200	Neg	Virus
A	80	40	20	15	7	7	<5	Alf/187
B	160	120	40	30	20	<5	<5	Alf/187
D	32	25	6	6	<5	<5	<5	Alf/187
E	200	50	12	12	<12	<12	<12	Alf/187
C	32	32	8	<8	<8	<8	<8	Alf
F	64	32	16	4	<2	<2	<2	GPE-
A	60	60	15	10	7	7	<5	CSF/Dieph
A	15	10	7	5	5	<5	<5	CSF/487/93(wb)
C	8	8	<8	<8	<8	<8	<8	CSFV/NADL
E	<12	<12	<12	<12	<12	<12	<12	CSF/Baker
A	<5	<5	<5	<5	<5	<5	<5	BVD/NADL
C	<8	<8	<8	<8	<8	<8	<8	BVD/NADL
B	<10	<10	<10	<10	<10	<10	<10	BVD/C24V
E	<12	<12	<12	<12	<12	<12	<12	BVD/Osloss
D	+	+/-	-	-	-	-	-	CSF/CTB ELISA

## Conclusions

1. The absence of cross reactions with BVDV is encouraging, but this needs further evaluation using strains of BVDV-type 2 and border disease virus.
2. Too high a dilution of positive serum was used in some of the mixtures, and new candidates should be prepared aimed at achieving a clear positive (regardless of the strain of virus used in the test) and a weak positive which scores consistently positive.
3. The strain of CSF virus used in the neutralisation test clearly has a critical effect on the results. There is a need for further international discussion on standardisation of this aspect of the test. Although European laboratories have largely standardised on Alfort/187, this has not been discussed in a wider forum.
4. Recipients of this report are invited to comment to the authors.

## SCIENTIFIC PAPERS ON CSF

### **Molecular characterisation of classical swine fever virus isolates collected in Belgium since 1988**

*H. Vanderhallen and F. Koenen*: Nationaal Instituut voor Diergeneeskundig Onderzoek, Ukkel, Belgium

#### Introduction

Molecular techniques, such as RT-PCR, for the detection of Classical Swine Fever (CSF) have been developed and their applicability in routine diagnosis is currently evaluated. Coupling RT-PCR with sequencing and subsequent phylogenetic analysis can give more detailed information about the spread and evolution of a series of CSF outbreaks. The value of this technique for the epidemiological analysis of CSF outbreaks has previously been demonstrated by Lowings *et al.* (1994).

The aim of our study was to characterise isolates collected shortly after the vaccination ban in April 1988 as well as isolates from the 1993-1994 and the 1990 epizootics in Belgium.

Two separate regions on the CSFV genome; the variable 5' part of the E2 gene (Lowings *et al.*, 1994) and the 3' end of the polymerase gene (Viçek *et al.*, 1996), were sequenced. Both approaches lead to the division of CSF viruses in two main groups consisting of five subgroups (Lowings *et al.*, 1996).

#### Material and methods

Original materials (tonsil or kidney) from the following outbreaks were analysed by the above mentioned methods. If no original material was available the corresponding cell culture supernatants were examined.

#### *Characteristics of the isolates*

- \* Isolate "Bassevelde/88" from an outbreak in 1988.
  - first outbreak 4 months after the vaccination ban was imposed in Belgium
  - several outbreaks in this region during vaccination
  - fatteners and sows were affected
  - problems in young piglets were recorded
  - lingering disease
  - origin unknown
  - characterised as highly virulent by experimental infection
- \* Isolate "Van Zele/88" from an outbreak in 1988.
  - from the same period and same area as isolate "Bassevelde/88"
  - characterised as mildly virulent by experimental infection
- \* Isolate "Wuustwezel/89" from an outbreak in the province of Antwerp in 1989.
  - several outbreaks in this region after the vaccination ban
  - lingering disease
  - origin unknown
- \* Isolates from the 1990 epizootic.
  - 2199/90
  - 2849/90
  - 2850/90
  - 1287/90
- \* Isolates from the 1993-1994 epizootic.

- The epidemiogram of outbreaks registered as contact infections is shown in Figure 1.
- Isolate H19/94 from an outbreak with unknown origin.
- Isolate H38/94 from a pre-emptively slaughtered outbreak. No clinical symptoms were evident and serology was negative at time of slaughter.
- Isolate H46/94 from last but one outbreak. Possibly due to persistently infected sows. The disease was clinically evident in weaned piglets.

### Results and discussion

The respective dendrograms are shown in Figures 2a and 2b.

Three isolates were collected shortly after the vaccination ban. Interestingly one of these isolates, "Bassevelde/88", clusters in group I together with historical CSFV isolates, vaccine strains, reference strain Alfort 187 and more recent isolates from Brazil and the Far East. This isolate seems different from all documented strains in this group. To confirm this clustering in subgroup I.1 an additional region on the viral genome was sequenced. The analysis of nearly the entire 5' NTR with primers developed by Hoffman (personal communication), confirmed the grouping (Figure 2c) and makes recombination events unlikely. "Bassevelde/88" was characterised as a highly virulent virus while "Van Zele/88", collected from the same region at the same time, exhibited low virulence. A possible hypothesis for the presence of this isolate in Belgium in 1988 is the introduction of a group I isolate by animal trade or by swill feeding of frozen meat. More molecular information on isolates circulating at that time will be necessary to trace back the origin of this outbreak.

All other isolates collected between 1988 and 1994 cluster in subgroup II.3. However, molecular variability was evident between the isolates from the respective epizootics. "Wuustwezel/89" is significantly different from isolates from the 1990 epizootic which confirms that this epizootic was due to a foreign introduction of CSFV in Western-Flanders and not linked to the outbreaks occurring in the region of Wuustwezel in 1989.

In contrast to the isolates from the 1993-1994 epizootic, which are identical, the isolates from the 1990 epizootic show some molecular variability in the 3' end of the polymerase gene. In 1993-1994 Belgium supplemented the stringent sanitary policy as imposed by directive 80/217/EEC with pre-emptive slaughtering. During the 1990 epizootic pre-emptive slaughtering was only started at the end of the epizootic. The approach adopted in 1993-1994 limited the unnoticed circulation of virus, allowing mutations to occur, by systematically eliminating all animals in direct danger of infection or already subclinically infected. ~~The presented results indicate that the sanitary policies imposed during a series of CSFV outbreaks can influence the epidemiology of CSFV.~~

### DISCUSSION

*Edwards:* Is it possible to get more information on the Bassevelde strain?

*Vanderhallen:* This is quite difficult.

*Paton:* Could it be a reversion of a C strain type? One could type it with C strain-specific mAbs.

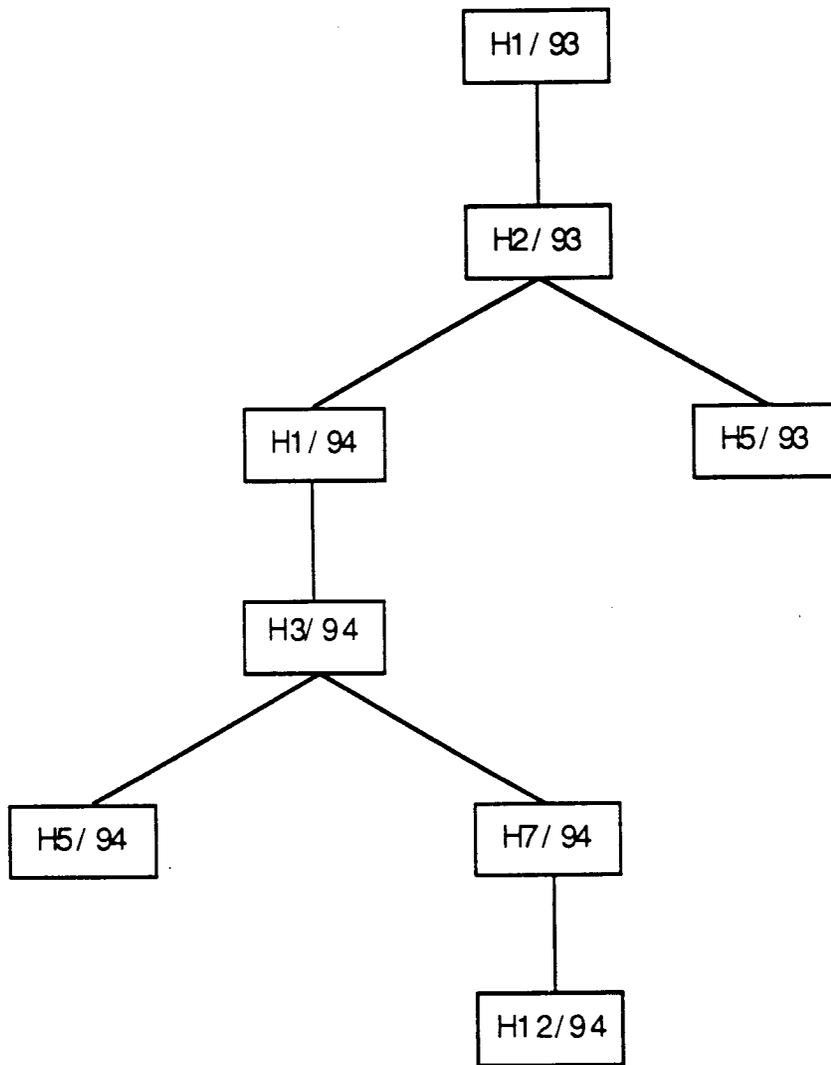
*Vanderhallen:* It reacts with mAb not reacting with C strain. This argues against reversion of C strain.

*de Smit:* How many isolates from 93/94 were analyzed?

*Vanderhallen:* Several selected isolates were sequenced, more are needed from the 1990 outbreaks

*Peysak:* How is CSF situation presently in Belgium?

*Koenen:* Free from CSF, but 25000 pigs have been killed due to preventive slaughtering. If NL farmers had CSF in NL, the pigs on the Belgian farm of the same owner are killed. Tight control on animal movements.



**Figure 1** Epidemiogram illustrating outbreaks of the 1993-1994 Belgian epizootic registered as contact infections



## The epidemiology of CSF in Germany between 1993 and 1997

*Jörg Fritzemeier, Irene Greiser-Wilke, Klaus Depner and Volker Moennig.* Institute of Virology, Hannover Veterinary School, Buenteweg 17, D-30559 Hannover, Germany

The high incidence of classical swine fever (CSF) in Germany as well as in other European countries in the present decade has once more demonstrated the great economic impact of this disease. Alone in Germany 315 outbreaks of CSF have been observed between 1993 and June 1997. In this study, epidemiological relations between outbreaks in different regions of Germany were analysed by molecular typing of CSF virus isolates. This method is based on phylogenetic analysis of defined regions of the genome and has been found to be an efficient extension to epidemiological investigations. Molecular typing can help to elucidate whether an outbreak was a single event only or if it was linked to other cases even in different geographic locations. The value of the results mainly depends on the universality of the data base containing the epidemiological information and the genomic sequences of CSF virus isolates from earlier outbreaks.

For genetic typing it was found that phylogenetic analyses of fragments of the 5' non translated region (5'-NTR), the E2- and the NS5B protein genes gave consistent results (LOWINGS et al., 1996). In the present study, phylogenetic analyses were performed comparing the 5'-NTR fragment between bases 136 and 408 (reference: Alfort-Tuebingen).

Phylogenetic analyses showed that the CSF virus isolates can be divided into two groups. The older isolates from before 1970 belong to group "1"; the newer isolates originating from Europe all belong to group "2". Group "1" isolates can be divided into subgroups "1.1" and "1.2" while in group "2" three subgroups ("2.1", "2.2" and "2.3") can be distinguished (LOWINGS et al., 1996). Each subgroup consists of a number of genetically closely related CSF virus isolates. Figure 1 shows a phylogenetic tree constructed from the 5'-NTR sequences of representative virus isolates characterised so far.

### ***Epidemic (1993 to 1995) in Rhineland-Palatinate, Baden-Württemberg and Lower Saxony***

The largest epidemic started in the south-west of Germany in Rhineland-Palatinate in the district of Germersheim in May 1993. The CSF virus isolated belonged to subgroup 2.3 and was designated as subgroup "2.3\*Spreda". Possibly a contact between infected wild boar and domestic pigs was responsible for the primary outbreak. The virus was spread by infected pigs delivered to Baden-Württemberg (8 outbreaks). Subsequently the disease was spread to Belgium and Lower Saxony. In Germany, 100 outbreaks of CSF could be attributed to this epidemic which lasted from May 1993 to November 1995. Subgroup "2.3\*Spreda" seems to be eradicated in Germany. Fortunately, this virus was not transmitted to wild boar in Lower Saxony.

### ***CSF in German wild boar***

In the east of Lower Saxony CSF virus subgroup "2.3\*Uelzen" is circulating in the wild boar population since summer 1992. From wild boar in Mecklenburg Western Pomerania CSF virus was isolated for the first time in autumn 1992. The infection extended to wild boar of Brandenburg at the beginning of 1995. In both provinces two virus subtypes ("2.3\*Rostock" and "2.3\*Güstrow") are predominant. Sporadically, the CSF viruses circulating in the wild boar population are isolated from domestic pigs in the corresponding regions. In most cases, the outbreaks were due to single events, sometimes followed by a limited number of secondary outbreaks. In these regions, it is conceivable that epidemics in domestic pigs may have been prevented mainly by the fact that their density is low. However, CSF in wild boar represents a

permanent danger for domestic pigs. The virus can be spread due to illegal swill feeding or contaminated vectors, or it can be transmitted directly by contact if domestic pigs are kept outside. The eradication of CSF in the wild boar population is an urgent common task.

*Virus types newly introduced into Germany since the end of 1996*

In a single outbreak in Lower Saxony the virus subgroup "2.2\*Ringelsdorf" was identified, which previously occurred in two wild boar in Austria last year. It is possible but not certain that there is a link between these outbreaks in Austria and Germany.

Furthermore the subgroup "2.3\*Kaernten" was newly introduced into a small holding in the province of Thuringia due to feeding of pigs with unheated kitchen waste. The same virus subgroup occurred before in Poland in 1992, in Switzerland in 1993 and 1994 (imported wild boar meat from Rumania) and in Austria in 1995.

The first outbreak in Germany in 1997 was observed in Northrhine Westfalia in the district of Paderborn. As before, it was also due to illegal swill feeding. The virus subgroup designated "2.1\*Pader" had been identified earlier in isolates from outbreaks in Bavaria (1989), in The Netherlands (1992), in Switzerland (1993) and also in Austria (1993) in illegally imported wild boar meat from China. The virus was spread to 23 holdings in the districts of Paderborn and Soest. At the beginning of 1997 CSF outbreaks occurred in The Netherlands followed by further outbreaks in Spain and Italy. Isolates of the outbreaks in The Netherlands, Spain and Italy were sequenced. Presumably these outbreaks are linked to the outbreaks in Northrhine Westfalia because they were also typed as "2.1\*Pader".

In summary, isolates of subgroups "2.1\*Pader", "2.2\*Ringelsdorf" and "2.3\*Kaernten" have been newly introduced into German domestic pig holdings. In two cases the herds became infected due to illegal swill feeding, and it can be assumed that the third case was due to the same route of infection. To avoid the import of new CSF viruses from other regions or countries, a strict control of the plants and pig holdings which have a licence to deal with swill or feed pigs with swill is necessary. More effort has to be made by the veterinary authorities to keep a public relation campaign running which clarifies the reasons for legislation concerning the use of kitchen waste as pig feed. On the other side the trade with contaminated meat is to be prevented. ~~In this aspect control measures at the Community's borders must become a more important duty in the future~~ in order to protect our holdings. In addition, inside the Community a reliable mutual information system will be the basis of an efficient control of CSF.

## REFERENCES

LOWINGS, P., IBATA, G., NEEDHAM, J. & D. PATON (1996). Classical swine fever virus diversity and evolution. *Journal of General Virology* 77, 1311-1321.

## DISCUSSION

*de Smit*: Which is the main way of spreading the virus?

*Fritzemeier*: Movement of infected pigs, vehicles, neighborhood, persons

*Moennig*: Differences of importance of factors between index cases and context cases. One has to focus on prevention of index cases (spread from WB, swill feeding, illegal import).

*Edwards*: Where is infected (WB) meat coming from?

*Moennig*: Possibly illegally imported WB meat as well as a lot of contaminated DP meat in circulation.

*Alemius*: Why does this happen only in Germany? More illegal swill feeding going on?

*Moennig*: Borders are wide open, not like e.g. U.S.A. Problematic swill feeders are not the big pig producers, but small farms (e.g. restaurants with own pigs).

*Buettner*: Indirect swill feeding (e.g. by dogs dropping bones in the pig stable) also occurs.

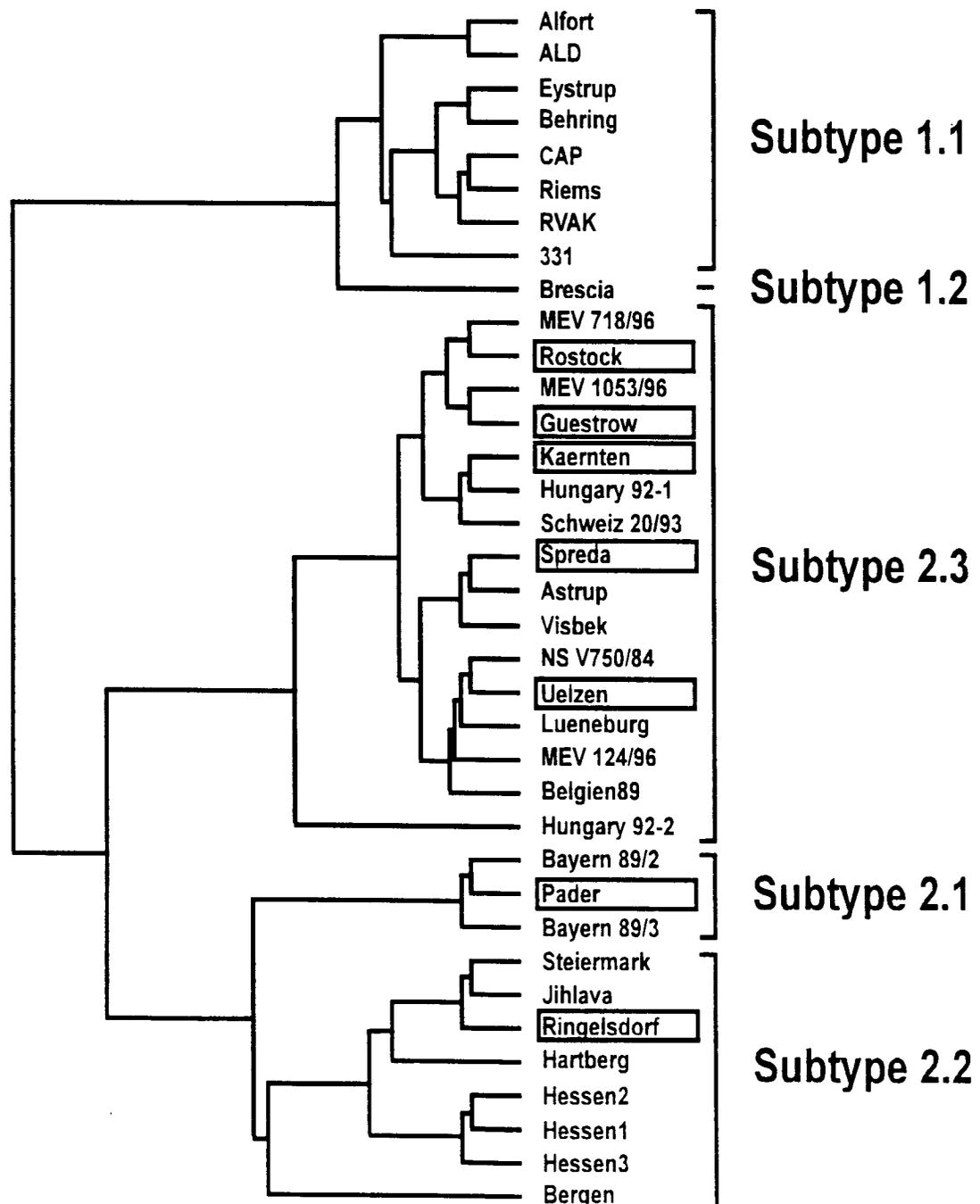
*Have*: Is swill feeding allowed by restaurants?

*Moennig*: No, restaurants may deliver swill to treatment plants, which then sell it to pig producers.

Not allowed to directly feed to own pigs. Pig producers only get license if swill is properly treated.

*Wilkinson*: A proper definition of swill is required. Weak point: own waste (crap) disposal.

**Figure 1:**  
Phylogenetic tree of the 5'-NTR sequences from representative CSF virus isolates. Sequence types of CSF viruses isolated in Germany since 1993 are framed.



## Molecular characterisation of recent European CSFV isolates

*Presented by Dr. Irene Greiser-Wilke*

*Veterinary School Hannover, Institute of Virology, Bünteweg 17, 30559 HANNOVER*

One of the aims of the cooperative CSF Fair project 3-CT95-0707 is the improvement of methods that allow the tracing of the spread of the disease by rapid typing of new isolates by molecular methods. For genetic typing, RNA is isolated from organ material or from cell cultures infected with the new isolates. Then, reverse transcription is performed, and selected regions of the genome are amplified by polymerase chain reaction (RT-PCR). The amplicons are sequenced, and evaluation of results is done by computer programs. These evaluations include sequence alignments and phylogenetic analysis using sequences from known isolates stored in a data base. Currently, three different sites of the CSFV genome have been selected for typing of CSFV isolates:

- Fragments of the 5' nontranslated region (5' NTR); (150 bp or 340 bp, respectively),
- A fragment of the E2-gene (190 bp)
- A fragment of the NS5B-gene (409 bp).

For evaluation of the method, we typed 57 CSFV isolates from recent European outbreaks by sequencing the three genomic regions. The isolates originated from outbreaks in Germany between 1984 and 1997 (27 isolates), from Austria between 1990 and 1994 (9 isolates), from Poland from the early 90-ties (11 isolates), from Hungary (1992, 2 isolates), from Italy (1991-1997, 4 isolates), and from Spain (1 isolate) and the Netherlands (1 isolate), both from 1997.

The phylogenetic study using 150 bases corresponding to the 5' NTR showed that all isolates analysed belonged to CSF group 2 (LOWINGS et al., 1996), including representatives from all three subgroups, namely 2.1, 2.2 and 2.3. Further discrimination was also possible, indicating that outbreaks within a given region were not always connected. In combination with known epidemiological data, genetic typing can greatly assist in tracing the course of the spread of the disease. An example is a series of epidemiologically linked outbreaks registered at the beginning of 1997, first in Northrhine-Westfalia (Germany), later in the Netherlands, and finally ~~in Italy and Spain. Viruses isolated from these outbreaks were shown to be very closely~~ related, within subgroup 2.1. It has now been clearly established that the primary 1997 outbreak in Germany was due to illegal swill feeding. Other viruses of this subgroup have been identified from Malaysia (in 1986), from Bavaria (in 1989), from the Netherlands (1992), from frozen wild boar meat imported from China into Austria (1993) and from Switzerland (in 1993 - HOFMANN, 1996).

With two exceptions, most Austrian isolates were of subgroup 2.2 and clearly different from recent German and Polish isolates, which were of subgroup 2.3. Other subgroup 2.2 viruses have been isolated from Italy between 1985 and 1991. Of the remaining two Austrian isolates, one originated from wild boar meat imported from Rumania (1994), while the other one was derived from a domestic pig (1995). These isolates were very similar to two Hungarian isolates from 1992.

The identical panel of isolates was typed using 190 bases from the E2 gene, and first results using the 409 bases from the NS5B were also available. Comparative phylogenetic analysis yielded the same results as for the 5' NTR, with no evidence of recombination between CSFV subgroups. Genetic typing using the E2 and the NS5B genes generally showed a higher resolution for strain discrimination (Fig. 1).

From these results we conclude that phylogenetic analysis with each of the three genomic regions leads to essentially the same grouping of CSFV isolates. The 5' NTR has a lower resolution, but is suitable for determining the subgroup of a new isolate. For fine discrimination of individual isolates, the E2- and / or the NS5B-genes should be used. Use of widely separated target regions guards against the possibility of mistyping due to recombination between viruses from different subgroups. However, our results have not provided any evidence that recombination of this nature occurs. An extensive database of sequences based on these three regions is now available and is being constantly enlarged. It is recommended that the same target regions should be used for sequencing new isolates, in order to standardise results and achieve comparability with the accumulated data (see Table 1 for PCR primers required). The results clearly show that genetic typing is a potent method for epidemiological studies.

This cooperative project includes results from the following partners:

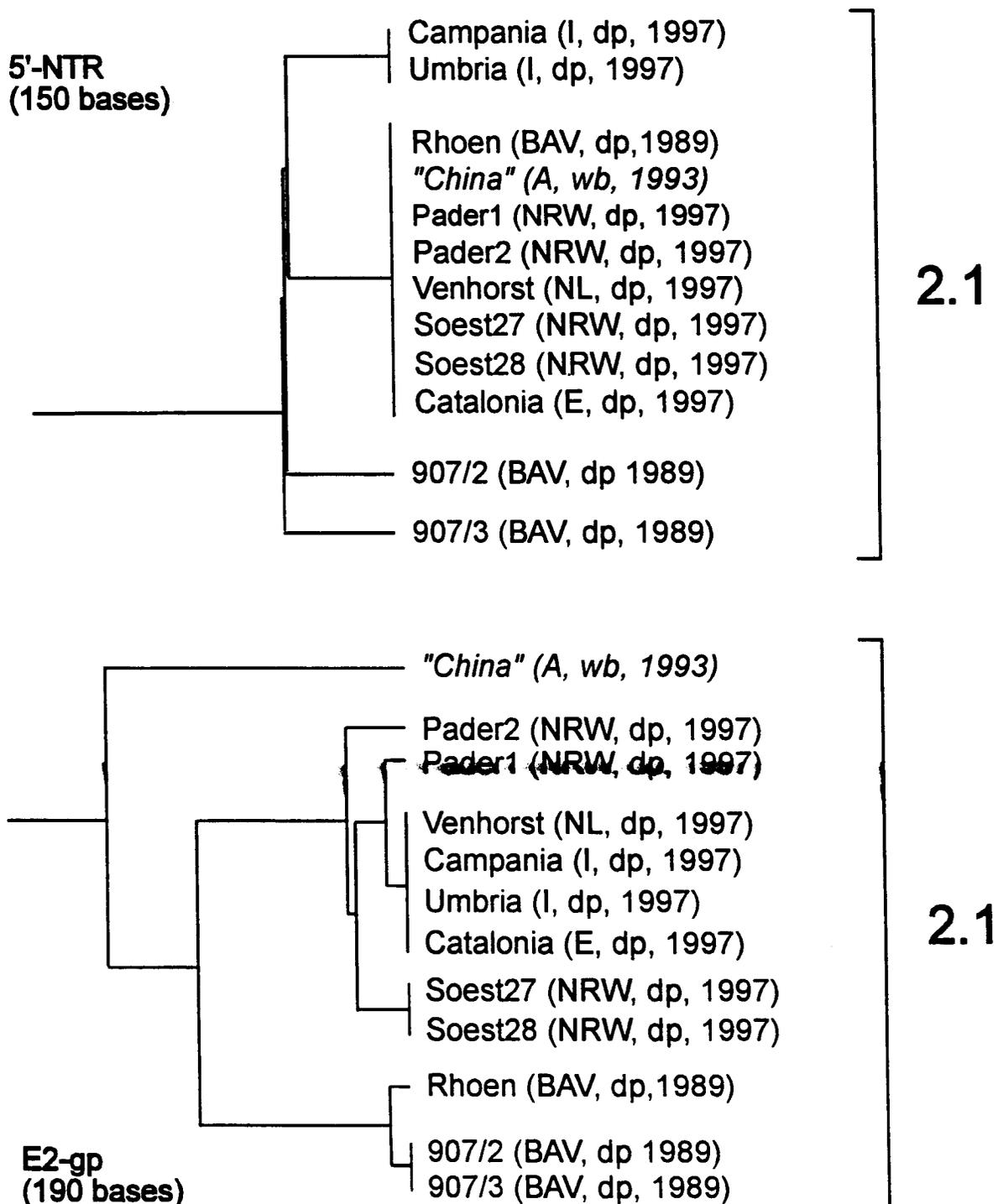
- The Central Veterinary Laboratory - Addlestone, Virology Department, UK;
- National Veterinary Institut - Uppsala, Department of Virology - Biomedical Center, Sweden;
- Nationaal Instituut voor Diergeneeskundig Onderzoek - Brussel, Dienst Varkensvirologie, "NIDO", Belgium;
- Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche - Prugia - I National Reference Laboratory for Classical and African Swine Fever, Italy;
- Hannover Veterinary School, Institute of Virology, Germany;
- The National Veterinary Research Institute - Pulawy, Swine Disease Department, Poland;
- Institute of Virology and Immunoprophylaxis, Switzerland.

FELSENSTEIN, J. (1989). Phylip: phylogeny inference package (version 3.5c). *Cladistics* 5: 164-166.

HOFMANN, M.A. (1996). Molecular epidemiology of CSF: Isolate Switzerland II/93 is closely related to a virus strain isolated from Chinese wild boar meat in Austria. Report on the Annual Meeting of National Swine Fever Laboratories. Alghero, Sardinia, Italy; 3 - 5 June 1996, p.p. 29.

LOWINGS, P., IBATA, G., NEEDHAM, J. & D. PATON (1996). Classical swine fever virus diversity and evolution. *Journal of General Virology* 77: 1311-1321.

Figure 1: Comparison of phylogenetic trees of fragments from the 5' NTR (top) and from the E2 protein gene of isolates originating from the outbreaks recorded 1997 first in Germany, then in The Netherlands, Italy and Spain, and from some isolates of the same Subtype from 1989 and 1993. Alignment of sequences was performed using CLUSTALW, and the trees were constructed using the DNADIST and the KITSCH programs from the PHYLIP-package (FELSENSTEIN, 1989)



dp: domestic pig    wb: wild boar  
 A: Austria        E: Spain        I: Italy        NL: The Netherlands  
 BAV: Bavaria     NRW: Northrhine-Westfalia

Table 1. RT-PCR primers recommended for use in future phylogenetic studies (Figures in parenthesis correspond to nucleotide positions in Alfort/187\* and Alfort Tuebingen\*\*, respectively)

5' NTR:

*Primers used for entire 5' NTR amplification of CSFV:*

CSF-R411 (antisense): 5' CAC TCC CAT TGG TTT TTG TTT GT 3' (411-433\*)

CSF-L001 (sense): 5' GTA TAC GAG GTT AGT TCATTC 3' (1-21\*)

*5'-IRD41-labelled sequencing primers used to sequence PCR products obtained with primers CSF-R411/CSF-L001:*

ACR408 (antisense): 5' CTC CCA TTG GTT TTT GTT TGT TTG 3' (408-431\*)

ACR119 (antisense): 5' GGC TAG TCC CTC CGT TTG 3' (119-136\*)

ACL072 (sense): 5' CTC CAG CGA CGG CCG AA 3' (72-88\*)

E2 gene:

*Outer set*

Forward 5' AGR CCA GAC TGG TGG CCN TAY GA 3' (2218-2240\*\*)

Reverse 5' TTY ACC ACT TCT GTT CTC A 3' (2888-2870\*\*)

*Inner set (for nested PCR and for sequencing)*

Forward 5' TCR WCA ACC AAY GAG ATA GGG 3' (2467-2487\*\*)

Reverse 5' CAC AGY CCR AAY CCR AAG TCA TC 3' (2738-2716\*\*)

NS5B gene:

S1 Forward 5' GACACGAGYGCAGGCAACA 3' (11128-11146\*\*)

S2 Reverse 5' AGYGGGTTCCAGGARTACAT 3' (11576-11557\*\*)

Same primers used for sequencing.

**DISCUSSION**

*de Smit:* Were the Bavarian 90 strains also due to connection to WB Austria/China?

*Moennig:* Probably not, the 93 China isolate is rather a reintroduction from somewhere.

*de Smit:* Which region of E2 was sequenced?

*Lowings:* N-terminal 190 bp

*de Smit:* Could differences result, if other (more) from E2 would be analyzed?

*Lowings:* There might be some more heterogeneity.

*Paton:* It is more important to realize that all sequences lead to similar results.

*Lowings:* Type 1 viruses are still present in other parts of the world (e.g. Mexico, Malaysia). The genotype distribution picture there might be different.

*Moennig:* Once worldwide picture is known, it is easier to follow CSFV spread.

*Belak:* Most viruses are type 2, which makes it easy to differentiate from vaccine strains. But: In Belgium type 1 isolate was found.

## Genetic heterogeneity of classical swine fever virus in Central Europe

*T. Stadejek, S. Vilcek, J.P. Lowings, A. Ballagi-Pordány, D.J. Paton and S. Belák  
Presented by Dr. Tomasz Stadejek: National Veterinary Research Institute, Swine Diseases  
Department, Partyzantow 57, 24-100 Pulawy, Poland*

The aim of this work was to characterize Central European isolates of CSFV and to evaluate the applicability of molecular epizootiology on a large number of viruses collected from recent outbreaks on a well defined geographic area.

Thirty four samples of virus infected cell culture supernatants or tissue samples from pigs and wild boar were used in the study. The samples were collected during the 1990s in Estonia (one sample), Hungary (5), Poland (16) and Slovakia (12 samples).

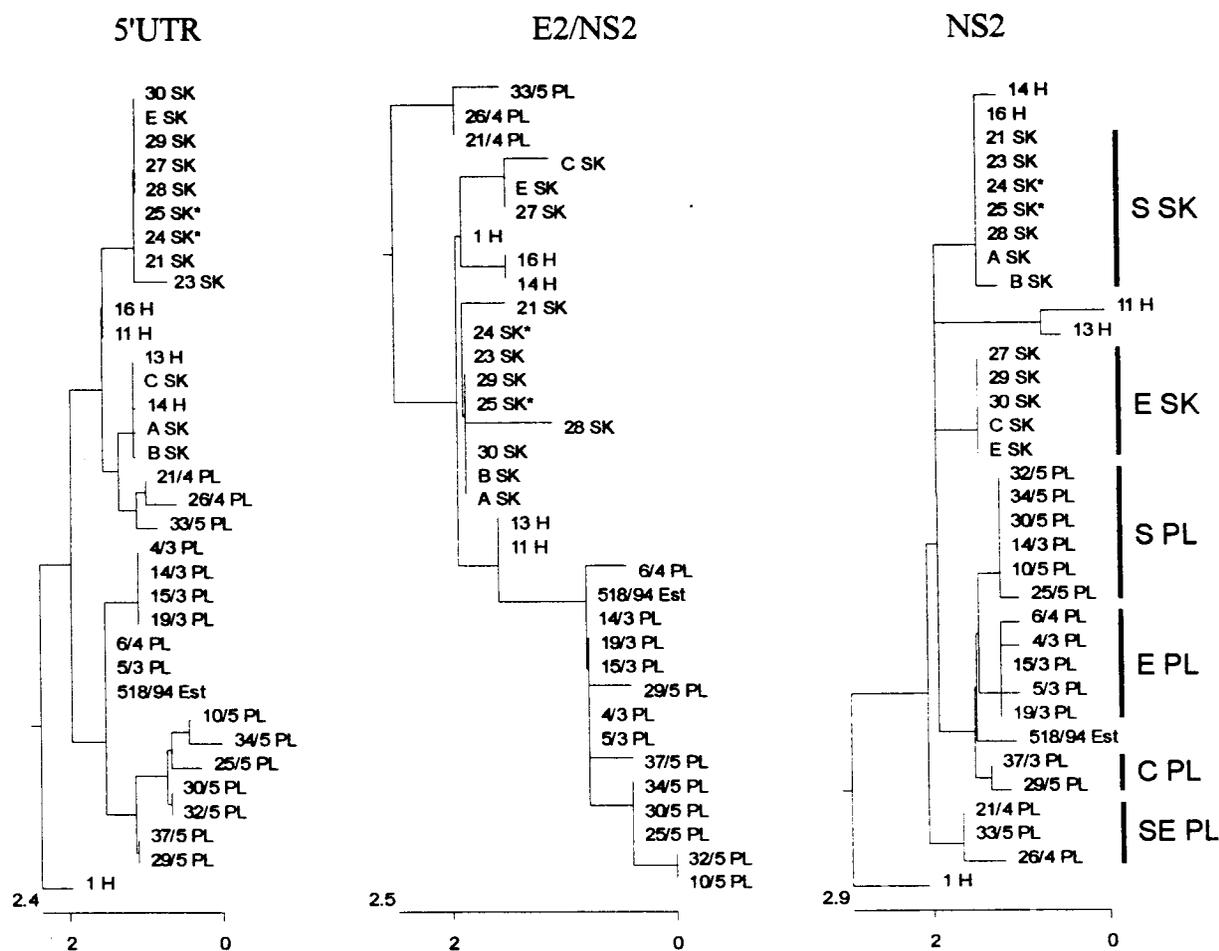
Primers were selected to amplify cDNA from three genome regions: 5'UTR, E2/NS2 and NS2. The PCR products were automatic cycle sequenced. Sequences of both strands were determined. Sequence alignments and phylogenetic analysis was done by computer programs.

Phylogenetic analysis of each of the three genomic regions taken in isolation produced three reproducible groups of viruses along with several disparate isolates (Fig. 1). These groups corresponded to defined geographical areas. One group included all the viruses from Slovakia and some from Hungary. Another group included only viruses from south eastern Poland and the last consisted of isolates from Estonia, south, east and central Poland. Examination of the terminal branches of the trees within these three main groups showed inconsistencies. Such inconsistencies were not surprising due to the small differences between isolates at this level. In some cases as little as one or two nucleotide substitutions were responsible for these local changes in morphology. The analysis of the NS2 tree showed the best correlation between morphology and the geographical origins of the viruses. Using this data it was possible to resolve six geographically distinct groups (Fig. 1). The Slovakian group was resolved into an eastern and a southern group whilst the central, eastern and southern Polish group was resolved into three subgroups corresponding to these areas. The south eastern Polish group also remained distinct as did the disparate isolates from Hungary. Only one virus (14/3 from east Poland) appeared to be grouped inappropriately with the viruses from south Poland. Four of the five viruses from south Poland were identical in the NS2 region as were five viruses from south Slovakia and two viruses from south east Poland. Analysis of the composite data improved resolution and showed all of the viruses to be distinct (Fig. 2). The morphology of the composite tree was almost identical to that of the NS2 region, however, the virus 14/3 was now appropriately grouped within the eastern Poland subgroup making the correlation between tree morphology and geographical distribution of isolates exact.

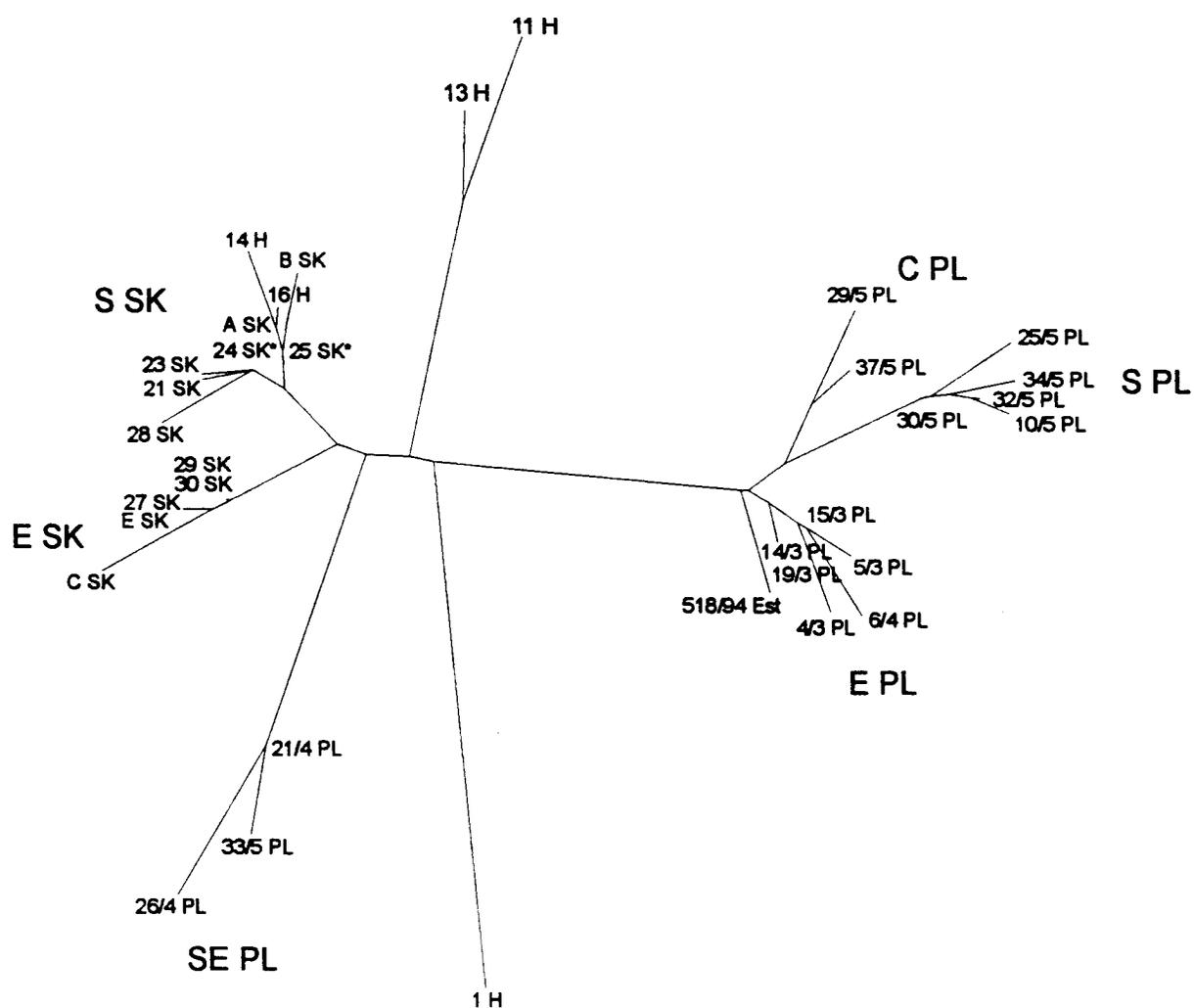
Whilst these data demonstrate the danger of over -interpreting small changes in limited regions of the virus genome they also emphasize that, with care, a genomic region can be selected where relatively few changes appear to correlate well with the known geographical distribution of isolates. Furthermore the resolution of this discrimination can be improved by including more sequence data into the analysis. In this study we have achieved this by the production of a composite dataset from three genomic regions. Thus, we could tentatively suggest that the isolates forming the four Polish groups are probably derived from different introduction events and are not the result of the spread of the same virus within Poland. The similarity of the Estonian isolate to the central, southern and eastern Polish viruses suggests that a more distant relationship exists in this case. A closer relationship can be established between some of the Hungarian isolates and those from south Slovakia. A more precise interpretation of the data would require the comparison of these sequences with a more extensive database of recently isolated European CSFVs in addition to the analysis of isolation dates and animal movements associated with these outbreaks.

CSFV infection in wild boar has been detected previously in several European countries. A study on epizootiology of CSFV in Sardinia proved wild boar to be reservoir of the CSFV. Wild boar were able to spread the virus even in the absence of contact with free-ranging domestic pigs. Genetic data obtained from isolates from Slovakia show that the same virus strain was circulating among wild boar and domestic pigs in this country. This observation strongly supports the important role of wild boar in maintaining the virus and in spreading the disease to domestic pigs.

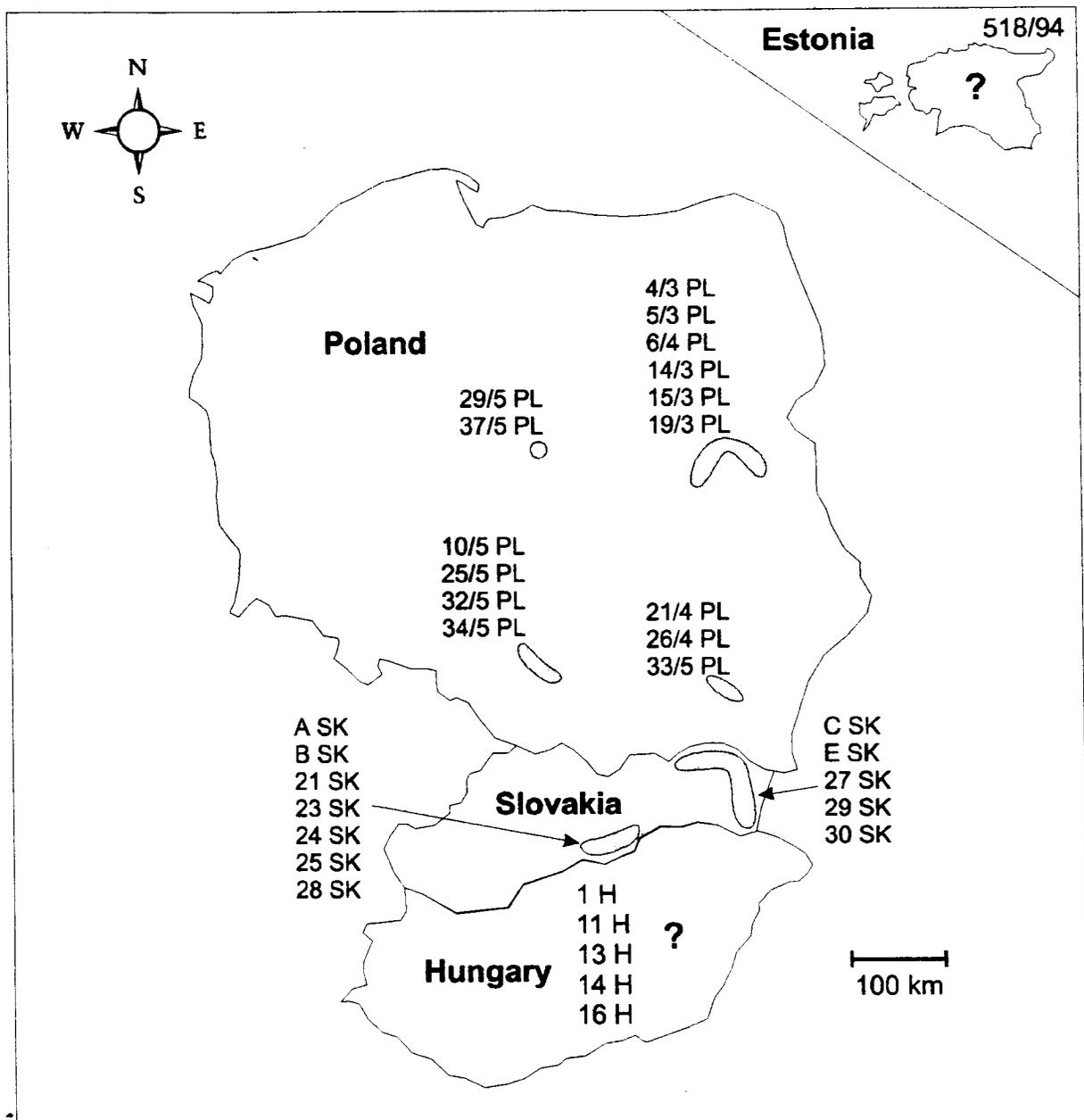
The study was supported by a grant no. 942529, from the Swedish Farmers Foundation for Agricultural Research, and partly by a grant no. PBO63/S3/94/06 from the Polish State Committee for Scientific Research, and by VEGA of SR (1/3001/96). This study has also benefited from collaboration supported by EU grant CT95-707.



**Fig. 1:** Phylogenetic trees of 34 strains from Central Europe constructed from: 232 nucleotides from 5'UTR, 254 nucleotides from E2/NS2 coding region and 415 nucleotides from NS2 coding region. The trees were constructed using neighbor-joining method. The length of each pair of branches represents the distance between sequence pairs. The scale beneath the trees measures the distance between sequences. Units indicate the number of substitution events. Isolates from wild boar are marked with an asterisk. Abbreviations: S south; E, east; C, central; SE, south-east; Est, Estonia; H, Hungary; PL, Poland; S, Slovakia



**Fig. 2:** Phylogenetic tree of 34 strains from Central Europe. The tree was constructed from nucleotide sequences of three genome fragments: 5'UTR, E2/NS2 and NS2 of total length 902 nucleotides, using Fitch-Margoliash method. The length of branches reflects phylogenetic distance relationship.



## DISCUSSION

*Wilkinson:* Why are geographically closely related viruses not mixed in Poland?

*Stadejek:* No idea, surprising result.

*van Oirschot:* What is the current CSF situation in Poland?

*Peysak:* Vaccination stopped in 1996, no CSF cases in DP in 1996, but in WB (10 cases). Reason: in 1996 most DP herds were heavily vaccinated immediately before vaccination ban.

**TaqMan: Automated PCR reading for CSFV diagnosis.**

*A. McGoldrick, P. Lowings and D. Paton*

The detection of CSFV and its discrimination from other pestivirus genotypes (BDV, BVDV I and BVDV II) requires relatively time consuming cultural methods or molecular analysis. In this study, a 5'-nuclease assay (TaqMan) was developed to enable the rapid distinction of CSFV from other pestiviruses through the use of a fluorogenic probe and with the minimum of downstream PCR processing. Based on the available sequence data, 5'NCR pan-pestivirus specific PCR primers were used in a one-step reverse transcription - PCR (EZ rTth RT PCR) to give an ~300bp fragment for all the 38 isolates tested. This product was subsequently used, in conjunction with a specific TaqMan CSFV probe, in a nested-PCR reaction with a second set of pan-pesti PCR primers (A11 and A14). During nested PCR, when the target of interest was present, the CSFV probe annealed to the amplicon between the forward and reverse PCR primers and was subsequently cleaved via the 5'-3' endonucleolytic activity of the DNA polymerase resulting in the release of the fluorescent reporter dye. Each PCR reaction tube was then placed directly into a Perkin-Elmer LS-50B Luminescence Spectrometer to monitor any increase of fluorescence due to the cleaved specific probe. Using this assay, we were able to rapidly distinguish representatives of each of the 5 known sub-groups of CSFV from all other pestiviruses. In addition, the TaqMan assay was shown to detect CSFV in infected pig blood with a sensitivity level equal to that of virus isolation.

**DISCUSSION**

*van Oirschot:* Is the sample preparation the same as so far?

*Lowings:* Yes. This is the next bottleneck to solve, e.g. in a 96 well format.

*van Oirschot:* What is the detection limit in terms of RNA molecules?

*Lowings:* Unknown.

*Grom:* Is this a complete robot for entire PCR?

*Lowings:* No, just for PCR reading, new version available for real time measurement of amplification.

*Vanderhallen:* Are the sequence differences between BDV and CSFV sufficient to allow differentiation by TaqMan?

*Lowings:* Differentiation works, solely based on specificity of the probe.

## Biological characterization of CSFV isolates from Sardinia

*De Mia Gian Mario, Feliziani Francesca, Rutili Domenico*

This work is part of the European Community research project on "control of CSF by molecular diagnosis and epidemiology" and consisted of characterization of the biological properties of virus isolates from sardinian outbreaks selected on the basis of their epidemiological relevance. Among the animal experiments, two of them were performed comparing groups of pigs with distinct genetic characteristics in order to determine if breed related factors influence the outcome of CSFV infection, as emphasized at the past meeting of SF laboratories. For this purpose different breeds of weaner pigs have been employed: 5 Large white and 5 hybrids (animal experiment 2); 5 Cinta Senese and 5 hybrids (animal experiment 3), being Cinta Senese an ancient Italian pig breed from Tuscany, mainly free reared and nowadays close to extinction.

### Animal experiment 2

Five weaner hybrid pigs and five Large White breed pigs were kept in separated pens. One animal/group was intranasally inoculated with 5 ml inoculum containing 300 TCID<sub>50</sub>/ml of CSF virus isolate 4618/Nuoro 96. All the tested animals presented a hyperthermic period: the five hybrids showed a thermal increase between day 17 and 23; the purebreed pigs between 7 and 15 days p.i. After a prodromal period, both groups presented the manifestation phase of the disease which started with body temperature > 41,0 °C and diarrhoea; very late during the terminal phase appeared cutaneous signs in 7 animals which consisted of haemorrhages. In this phase all the pigs had severe pulmonary symptoms as well as nervous symptoms including incoordination of movement and paresis. One pig (purebreed) which did not show nervous signs survived the acute phase of the disease becoming convalescent. All the others died, the hybrids between 28 and 42 days p.i., the purebreed pigs between 19 and 30 days p.i. At post mortem examination six pigs had some cutaneous erythema- all but two animals had severe hypertrophy and haemorrhages of lymph nodes. Petechial haemorrhages were present on renal cortices (7 pigs), serosal membranes (8 pigs) and muscles (4 pigs). Only one pig presented spleen infarction, In two pigs there were no acroscopic abnormalities apart from haemorrhages on serosal membranes and lungs.

### Animal experiment 3

Five weaner hybrid pigs and five Cinta Senese breed pigs were maintained in two separated pens. One pig/group was inoculated intramuscularly with 2 ml spleen extract containing 500 TCID<sub>50</sub> of CSF virus isolate 4691/Nuoro 96. All the animals presented a thermal increase: the first was the pig inoculated, on day 7; the remaining between 16 and 37 days p.i. Seven animals developed clinical evidence of swine fever and died, the hybrids between 42 and 58 days p.i., the purebreed pigs between 21 and 50 days p.i. Clinical findings were similar to those described previously with the exception of the nervous symptoms which were absent, Two pigs (purebreed) survived becoming convalescent. At necropsy 5 pigs had some cutaneous erythema; all but two pigs had mild to severe hypertrophy and haemorrhages of lymphnodes. Three pigs presented spleen infarction. Petechial haemorrhages were present on renal cortices (4 pigs), gut (3 pigs) and muscles (2 pigs). Five animals showed lesions such as "button" ulcers in the ileocaecal valve.

### Discussion

Nine out of ten (animal exp. 2) and eight out of ten weaners (animal exp. 3) developed the sub-acute form of CSFV infection on the basis of seventy of clinical signs and post mortem lesions. However, taking into account the duration of the disease, it should be classified as a sub-acute to chronic form of infection. A great similarity between individual reactions regarding the clinical signs during the prodromal and manifestation phase was noted between the two groups (hybrid and

purebred) of animals. A striking difference was noticed when their incubation periods were compared, as judged from the onset of fever and viraemia. This might be an indication for breed-dependent variations in the susceptibility to CSFV. In addition, it is noteworthy that in both experiments only purebred pigs survived the acute phase of the disease becoming convalescent. That could be due to differences of individual reactions as well as it might be an indication for breed-dependent variations in the immune response. The clinical evolution was typical of the disease but it is likely that under field conditions the virus dose would have been lower and therefore the outcome of the disease might be less clear in some individuals than in the experimental animals. Finally, it is noteworthy that no single clinical or post mortem feature made it possible to differentiate this condition with certainty from African swine fever which is also present in Sardinia.

#### DISCUSSION

*Depner*: Own findings are opposite: Incubation similar, but then different outcome of the disease afterwards. Also only short time of viraemia observed.

*van Oirschot*: Difference in immune response between purebred and crossbred pigs? Different antibody titers?

*de Mia*: No differences observed.

#### **Classical swine fever virus (CSFV) transmission by artificial insemination**

*A.J.de Smit, A. Bouma, J. van Oirschot, C. Terpstra*

During the current outbreak of Classical swine fever (CSF) in the Netherlands, CSFV has been introduced into a pig artificial insemination station (AIP). This was the first time in the history of CSF in the Netherlands. Movement restrictions and detection of CSF specific antibodies in serum samples from boars present on the station indicated that virus was introduced at least four weeks before CSF was diagnosed. During that period semen collected from boars on the AIS was distributed to hundreds of pig holdings.

The impact on the spread of CSFV was difficult to predict due to several factors, one of these was the lack of data concerning the excretion of CSFV into semen and the infection of CSFV by artificial insemination (or the genital route). Therefore the following animal experiment was undertaken to provide more data on these issues.

Three conventional raised boars, free of pestivirus antibodies, were trained to ejaculate after mounting a dummy. On day zero they were inoculated by the intranasal and intramuscular route with a CSFV isolate from the current outbreak (NL97/01). The ejaculate collected from each service was treated (e.g. diluted) for artificial insemination. From day 5 till 19 after inoculation, sows (in heat and free of pestivirus antibodies) were inseminated with semen from the infected boars and housed individually.

Up till now the following preliminary results were obtained. During the experiment the boars and sows did not develop any clinical signs typical for CSF (e.g. fever). CSFV was isolated from the semen of one boar during a short period after inoculation. So far the boars and two inseminated sows have developed antibodies against CSFV.

The toxic effect of semen on cells used for virus isolation precludes the conclusion that virus was absent in semen samples from the remaining two boars. Therefore collected semen samples will be tested more extensively for the presence of CSFV. It cannot be excluded that more sows will seroconvert for CSFV in the coming 14 days.

The results from this experiment demonstrate the transmission of CSFV infection by artificial insemination. Other experiments will be necessary to assess the probability of CSFV infection by the genital route.

#### DISCUSSION

*Alenius*: Why infected both orally and i.m.?

*de Smit*: Wanted to be sure that animals get infected

## Detection of CSFV in muscle samples from experimentally infected pigs

*Barbara Thuer*

Four CSF outbreaks in 1993 in Switzerland were presumably caused by feeding not properly heated swill. Molecular epidemiological data suggest that contaminated meat imported from other countries was the source of the CSFV infection. The aim of our investigations was to find a suitable method for CSFV detection in skeletal musculature of infected pigs in order to allow a routine testing of imported meat for CSFV contamination.

Using samples from 14 specific pathogen-free pigs, experimentally infected with CSFV strains Alfort/187, Brescia or Eustrup, respectively, the sensitivity of virus detection in meat was compared with the detection in target organs. Although both cell culture isolation as well as reverse transcription polymerase chain reaction (RT-PCR) are sensitive methods for CSFV detection in target tissues, muscle samples yielded unsatisfactory results.

		musculature	target organs
RT-PCR	positive	6	10
	negative	8	4
cell culture	positive	6	10
	negative	8	4
immunohisto-chemistry	positive	0	9
	negative	14	5

58% of investigated muscle samples were positive by RT-PCR, 38% were positive by virus isolation. RT-PCR with previous RNA extraction was much more sensitive than without. Ultracentrifugation and sonification of samples before RT-PCR did not result in higher sensitivity. There was no obvious correlation between bleeding of the animal and CSFV detection in musculature. However, a correlation between virus strain, severity of illness and CSFV detection in musculature was seen. The highly virulent strains led to much better results. In Alfort/187-infected pigs with only mild symptoms CSFV in general could not be detected. Therefore, for a reliable diagnosis of CSFV infection it is mandatory to examine other organs than musculature, such as tonsils, lymph nodes or kidneys. Exclusion of CSFV infection based on virus detection in skeletal muscle samples is unreliable.

## DISCUSSION

*R. Krassnig* asked whether there was any relationship between virus distribution and muscle type.

*B. Thür* said that no correlation had been seen

*M. Büttner* suggested that it would be difficult to detect virus in a large batch of meat as only a small amount may be contaminated and the sample size taken for PCR is small.

*B. Thür* agreed.

## **CSFV detection in blood from experimentally infected pigs**

*M. Hofmann*

In order to determine the optimal way to collect blood samples from CSFV-infected animals for virus detection by RT-PCR and cell culture, EDTA, heparinized and citrate blood as well as serum and clotted blood samples were tested for their suitability. It was shown that all the blood samples allowed a virus detection by both assays 3 to 4 days p.i. from CSFV Alfort/187-infected pigs.

Next, a clinical score list was developed to allow a quantitative judgement of the disease severity of CSF. The following parameters were scored from 0 to 3 and then added to a total daily disease score: Liveliness, body tension, body shape, breathing, walking, skin, eyes, appetite, defecation, leftovers in feeding trough.

Subsequent animal experiments were carried out to compare the in vivo properties of the recombinant vA187 CSFV with its parent strain Alfort/187. The recombinant virus showed the same properties as Alfort/187. Another experiment was aimed at comparing the Alfort/187 strain with the two highly virulent strains Brescia and Eystруп. It was found that Eystруп and Brescia were indeed highly virulent, leading to acute CSF, whereas Alfort/187 under our experimental conditions (SPF animals) produced almost no clinical symptoms.

A third experiment using both SPF and non-SPF animals and Alfort/187 was performed to determine the influence of the hygiene status of the pig on the outcome of the disease. Again SPF animals as in previous experiments did not show distinct disease signs, non-SPF animals on the other hand developed typical acute CSF, but recovered as well. Therefore it could be demonstrated that environmental conditions have a significant influence on the clinical manifestation of CSF.

## **DISCUSSION**

*J van Oirschot* stated that any scores should be carried out by "blind testers" as prior experience would have an influence upon the results. He also stated that the weight of pigs or food uptake should be used as a scoring criteria.

*M. Hofmann* replied that limited staff numbers in the high security laboratory meant that regular "blind" testing was impossible. A "blind" test trial had given similar results to those obtained by trained personnel. Food was considered in the scoring criteria in a semiquantitative manner as appetite was assessed and food remaining was weighed. Measurement of pig weight fluctuation was thought to be inappropriate due the short time span of the experiments

## First results of the surveillance programme for CSF in wild boar in defined areas of Austria

*R. Kraßnig*

650 wild boar in special districts of Lower Austria (north of the river „Danube“ and the district Bruck/Leitha) and the Federal state of Burgenland should be investigated.

Hunters are paid (300 ATS) for taking samples (organs and blood).

State veterinarians send the samples to our institute.

This programme was started in December 1996 and is supported by the Commission.

### Material of 277 wild boar (until 4.6.1997)

Spleen: 275

Kidney: 276

Lymphnode: 251

Tonsil: 150

Blood sample: 262

Organ fluid: 8

Organ samples of 4 wild boar were not investigated: autolysis.

### Some facts about the shooten/dead found wild boar:

Age	male	female	unknown
< ½ y	16	12	7
½ - 1 y	29	23	-
1 - 2 y	60	48	5
2 - 3 y	20	13	1
3 - 4 y	16	4	1
4 - 5 y	7	1	-
> 5 y	2	2	-
unknown	-	1	-
unknown	-	-	9

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### Method:

Organ samples: DIF in cryocut sections and Ag- ELISA (Hoechst)

Blood samples/ organ fluid: CTB- ELISA (Lelystad) and Ag- ELISA (Hoechst)

### Results:

1) Until today we didn't find positive wild boar in the regions Korneuburg, Mistelbach, Hollabrunn, Horn and Zwettl which were infected in the years 1990 - 1993.

Also we did not find positive ones in other regions.

2) In this statistic we've excluded the special area of Drösing and Ringelsdorf (district Gänserndorf), because there we found CSFV-antibodies in some wild boar in 1996 and 1997.

3) One wild boar, a four month old male (Burgenland, Neusiedl a. See) reacted doubtful in the CTB- ELISA. Retesting in the NIFT was impossible, the blood sample destroyed the cells (toxic reaction). Organs and blood were negativ for CSFV.

All the other 14 wild boar samples from the same region were CSFV and - antibodies: negative.

### Summary:

Screening is still going on, but at the moment it seems to be quiet in the northern parts of Lower Austria and Burgenland.

## DISCUSSION

*T. Müller* expressed concerns that the number of samples taken were small compared to the geographical area studied.

*R. Krassnig* agreed but suggested that positive samples had been found in previous surveys of this scale in the area examined and in this survey all samples were negative for antibody and virus.

*S. Alenius* asked which Ag ELISA was used.

*R. Krassnig* replied that the Bommeli ELISA was used and that tissue culture was also performed on the first 30 samples.

*W. Schuller* stated that it was sometimes difficult to detect virus in wild boar samples and that perhaps we should look at using PCR.

*J. Holejsovsky* stated that some wild boar samples seem to have a toxic effect on cell cultures and that bone marrow samples were used in his laboratory for virus isolation.

*W. Schuller* said that, in Austria, this would cause problems as most sampling is carried out by hunters.

### **BVDV- investigations: Contact of a cow with a sheep and a pig**

R. Kraßnig, A. Loitsch and M. Al-Nuktah

**Animals: Cow:** 11/2 year old, since one year at our institute. Status: PI= persistent BVDV-infected animal (genotype 1);

**Sheep:** Three year old wether from our institute own sheep flock. Status: BVDV (SNT) 1:16, virusisolation: negative.

**Pig:** Piglet, 20kg, castrated male. Status: BVD- and CSF- negative.

**Contact:** direct contact: „nose to nose“

#### **Method:**

**CSF- virus:** Antigen (Ag)- ELISA: Hoechst

- **antibody:** NIFT: Alfort 187, 100 KID50; PK (15) and CTB-ELISA (Lelystad)

**BVD- virus:** cow: Ag- ELISA (Dr. Bommeli); sec. bov. lung cells, PCR

(BA- Linz); Sheep and pig: MDBK- and sec. bov. lung cells.

- **antibody:** SNT; strain: Singer, 100 KID 50; MDBK- cells.

#### **Summary:**

**Pig:** The piglet developed antibodies against BVDV **five weeks** after contact with the cow.

Ten weeks after seroconversion, cross reaction with CSFV was detected in the NIFT, but only for the period of two weeks. The highest antibody- titer against BVDV was 1: 45.

The CTB- ELISA and the CSF Ag- ELISA always reacted negative.

**Sheep:** The sheep already had had „BVDV-antibodies“ before we started this trial. After 13 days of contact it seemed that the sheep had been boosted. But this picture was not so clear. We didn't see the higher antibody- titers for a long time.

**The daily taken body temperature of the pig and the sheep never gave an indication for the beginning of an infection. Clinical Symptoms never were seen.**

**Cow:** The cow had been tested for BVDV-antibodies sporadically. It has always been negative. BVDV has always been found in taken blood samples.

Start: 8.1.1997		PIG			SHEEP			COW	
Date	ESPV		BVDV		BVDV		BVDV		
Sample	Ag	NIFT	CTB-Eli.	SNT	Ag	SNT	Ag	SNT	Ag
15.1.	neg	neg	neg	neg	n.d.	1: 23	neg	neg	<b>pos</b>
21.1.	neg	neg	neg	neg	n.d.	<b>1: 91</b>	neg	n.d.	n.d.
29.1.	neg	neg	neg	neg	n.d.	<b>1: 64</b>	n.d.	n.d.	
7.2.	neg	neg	neg	neg	neg	<b>1: 181</b>	neg	n.d.	n.d.
14.2.	n.d.	neg	neg	<b>1: 8</b>	neg	<b>1: 91</b>	neg	neg	<b>pos</b>
21.2.	neg	neg	neg	<b>1: 2</b>	neg	<b>1: 64</b>	neg	n.d.	n.d.
28.2.	n.d.	neg	neg	<b>1: 2</b>	n.d.	<b>1: 32</b>	n.d.	neg	n.d.
18.3.	n.d.	neg	neg	<b>1: 8</b>	neg	n.d.	n.d.	n.d.	n.d.
11.4.	neg	neg	neg	<b>1: 11</b>	n.d.	<b>1: 45</b>	neg	n.d.	n.d.
18.4.	neg	neg	neg	<b>1: 23</b>	neg	1: 23	neg	n.d.	n.d.
25.4.	n.d.	1: 22	neg	<b>1: 23</b>	neg	1: 16	neg	n.d.	n.d.
5.5.	neg	1: 4,5	neg	<b>1: 16</b>	n.d.	1: 32	n.d.	neg	<b>pos</b>
23.5.	neg	neg	neg	<b>1: 45</b>	n.d.	1: 32	n.d.	n.d.	n.d.
2.6.	neg	neg	neg	<b>1: 32</b>	n.d.	1: 45	n.d.	n.d.	n.d.

n.d. = not done

#### DISCUSSION

*I. Greiser-Wilke* suggested that the virus present in the PI cow should also be assessed.

*R. Krassnig* agreed.

## Nomenclature for pestivirus isolates

(D. Paton)

There has been much debate on the classification of pestiviruses. It has become increasingly apparent that the host-based nomenclature for the different virus species is inappropriate and the latest recommendation to the International Committee on the Taxonomy of Viruses reflects this. Accordingly, the four principal pestivirus genotypes are to be given a numeric identifier - pestivirus 1-4. Pestivirus 1 equates to bovine viral diarrhoea virus-1, BVDV-1; pestivirus 2 equates to bovine viral diarrhoea virus-2, BVDV-2; pestivirus 3 to border disease virus (BDV); and pestivirus 4 to classical swine fever virus (CSFV).

What is still required, is a standard and informative system for naming individual isolates. Presently there is no accepted approach, and some isolates have names, others numbers and others combinations of both. Frequently the nomenclature used has no meaning except to the person assigning it. With ever larger numbers of isolates being characterised and described, the situation is becoming increasingly confusing. Sullivan et al. (1997) have suggested that the system of nomenclature for influenza viruses could be adopted for use with pestiviruses. With this approach a virus is named according to the virus species, the host species from which it was isolated, the country of origin and the year of isolation. It is also given a numeric identifier. Thus: virus spp. / host spp. / country / number / year; e.g. A / swine / England / 195582 / 92.

For the pestivirus equivalent: The virus species should adopt the newly accepted taxonomy i.e. Pesti 1-4. Where the genotype is uncertain the number can be omitted. The host species can be identified by two or more letters: i.e. Po = porcine, wPo = wild boar, Bo = bovine, Ov = ovine, Ca = caprine, Ce = cervine, wR = wild ruminant. The country can be identified by the international one or two letter code: e.g. D = Germany, Ch = Switzerland, F = France. An additional county identifier or equivalent would be useful and optional. The unique identifier should identify the institute where the isolation was made as well as a number to indicate which particular isolate is being referred to: e.g. Wey23 (the 23rd isolate made at the Central Veterinary Laboratory, Weybridge). The year of isolation can be abbreviated to two numbers. Thus: Pesti-4 / Po / F / Alf187 / 66 = CSFV strain Alfort 187. This system would be particularly recommended for use in official documents and published papers.

### Reference

Sullivan DG, Chang G-J, Akkina RK (1997) Genetic characterization of pestiviruses: sequence analysis of viral genotypes isolated sheep. *Virus Research* 47, 19-29.

### DISCUSSION

*Alenius:* What about China/Austria case? Which country should be taken?

*Paton:* Country of origin is important, not where virus was isolated.

*Moemig:* Finer resolution is needed, e.g. by including Bundesland, not only Germany.

*Paton:* This is already done for influenza (e.g. state within the U.S.).

*Moemig:* FAIR group should forward nomenclature proposal to EU commission on CSF.

## **Pestivirus contamination of bovine fetal serum: still a problem ?**

*M. Büttner*

It is well known that bovine fetal serum (FBS) can be contaminated by bovine viral diarrhea virus (BVDV) (Nuttal et al, 1977). Not long ago the contamination mostly consisted of viable virus. Thus FBS was an important source for unwanted BVDV infection of highly permissive cells and cell lines of ruminant origin. Moreover even cell lines derived from other species (cat, rabbit, monkey) can become infected and stay infected via pestivirus contaminated FBS (Bolin et al., 1994). Recently the contamination of human virus vaccines with pestivirus RNA has been described (Harasawa and Tomiyama, 1994). Due to the possibility of enormous heterogeneity of distribution of a virus (BVDV) contaminant in large batches of FBS the probability of viable virus to become overt increases when high volumes of FBS have to be used for cultivation of bulk cell cultures. Different techniques (e.g. ultra-centrifugation, cross-flow membrane separation) can be applied to concentrate the contaminating virus and such improve the rate of recovery. Viable virus may be detected using classical methods. We recommend incubation of pestivirus-free highly permissive cells for 4-5 days and the analysis of the cells with pan-pestivirus reactive - or BVDV-specific antibodies by indirect immunofluorescence and the aid of flow cytometry. The failure of detection of a virus replication, however does not exclude FBS contamination. Since nowadays FBS usually is treated by irradiation the capability of infection of contaminating BVDV is most likely to be destroyed. However, irradiation does not necessarily affect the integrity of viral RNA. We have applied a RT-PCR and nested PCR in the 5' non-coding region of the pestivirus RNA (Vilcek et al., 1994). The primer positions in the genome of BVDV NADL are given as follows: primer 324: position in NADL 108-128 PCR product: 288 bp primer 326: position in NADL 395-375 nested PCR product: 196 bp.

After phenol/chloroform RNA extraction from 6 native FBS samples (100 ml) of different producers and from one so called „serum replacing supplement“ pestivirus-specific cDNA was amplified from all RNA samples after application of the nested PCR protocol. Two RNA samples yielded a specific cDNA product (288 bp) already after the RT-PCR procedure. RNA extracted from three out of four different batches resulted in pestivirus-specific positive RT-PCR products. The conclusion drawn from these results is that the detection of pestivirus RNA by RT-PCR amplification of conserved sequences in the 5' non-coding region can become problematic when pestiviruses have been propagated in cell cultures maintained in FBS containing media. Even PK-15 cells grown in medium supplemented with irradiated FBS gave positive results in the described RT-PCR. At the moment it is not clear whether the whole genome of pestiviruses (namely BVDV) remains intact in FBS after irradiation or by chance especially the 5' non-coding region is not affected at least the sequences targeted by the PCR developed by Vilcek et al.

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- Vilcek, S., Herring, A.J., Herring, J.A., Nettleton, P.F., Lowings, J.P., Paton, D.J. (1994) Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. *Arch Virol* 136: 309-323

## DISCUSSION

*B. Thür* asked if antibodies were detectable in any of the samples of FCS.

*M. Büttner* said not

*V. Moemig* said that the lack of detectable antibodies or virus was not surprising due to the large number of animal that contributed towards the pool but such low levels still present a problem for PCR

*S. Alenius* said that they avoid problems by checking individual fetuses by PCR but also noted that FCS from Finland should be negative by PCR.

*K. Depner* cautioned that testing by PCR may not detect low level of virus in large volumes due to the small sample size used.

*M. Büttner* agreed that the FCS sample requires concentrating prior to PCR.

*M. Hofmann* suggested that if the extent of viral RNA degradation was to be determined from the FCS samples, the amplification of longer regions of the genome should be attempted.

*W. Schuller* asked how the participants inactivated their FCS. Answers ranged from use of Beta propiolactone, selection of antibody negative animals followed by irradiation and testing on tissue culture, PCR, use of FCS from Finland and the avoidance of FCS by adapting cells to grow with horse serum.

*S. Belak* brought up concerned about pestivirus infection in humans

*V. Moemig* stated that pestiviruses don't replicate in human cells

*J. van Oirschot* stated that even in potentially high risk groups there was no evidence for seroconversion to BVDV or CSFV

## SCIENTIFIC PAPERS ON ASF

### Assembly of African swine fever virus

*Thomas Wileman, Isabelle Rouiller and Christian Cobbold:* Department of Immunology, Pirbright Laboratory, Institute for Animal Health, Ash Road, Pirbright, Woking, Surrey, England

Presented by *Philip Wilkinson*

African swine fever (ASF) virus is a large (dia. 200nm) icosahedral enveloped DNA virus. The mechanism of assembly of the viral particle, and the origins of the membrane envelopes of ASF virus are largely unknown. In this study a monoclonal antibody recognizing p73, the major structural protein of ASF virus, was generated to analyse the binding of p73, a protein that lacks membrane targeting sequences, to membranes during the assembly of the virus. Approximately 50% of the intracellular pool of p73 associated with endoplasmic reticulum (ER) membranes as a peripheral membrane protein. Binding was rapid and complete within 15 minutes of synthesis. A protease protection assay demonstrated a time-dependent envelopment of the membrane-bound, but not cytosolic, pool of p73. Envelopment of p73 took place one hour after binding to membranes and was completed within two hours. These results provide evidence for the binding of ASF virus structural proteins to the ER and implicate a role for the ER in envelopment of ASF virus.

The order of events producing icosahedral ASF virions is not known, but there are two main mechanisms for the assembly of icosahedral ASF virus particles: it could take place in the cytosol prior to envelopment or on ER membranes during envelopment. To distinguish between these two pathways, the state of oligomerization of p73 associated with cytosolic and membrane fractions was assessed by sucrose density sedimentation. The results showed that p73 molecules assembled into dimers immediately after synthesis in the cytoplasm. Significantly, during a pulse-chase analysis the ER-associated pool, but not the cytoplasmic pool, formed large oligomeric complexes in excess of  $20 \times 10^6$  Da. The kinetics of complex formation closely matched that of envelopment. Drugs added to cells that inhibited envelopment also prevented oligomerization of p73. These results suggest that assembly of icosahedral ASF virus particles takes place on the ER membrane and is functionally linked to the wrapping of capsids by ER cisternae.

#### Reference

Cobbold, C., Whittle, JT., Wileman, T (1996) Involvement of the endoplasmic reticulum in the assembly and envelopment of African swine fever virus. *Journal of Virology*, 70, 8382-8390.

## REPORT ON WORK RELATED TO CSF CARRIED OUT BY THE SUB-GROUPS OF SCIENTIFIC VETERINARY COMMITTEE.

(Chairman: J Westergaard, Rapporteur: D Paton)

### Minimum requirements for NSFL

*S Edwards, K. Depner, F. Koenen, T. Mueller*

*Chairman:* The need for this guidance was discussed at the last meeting in Sardinia. A small working group had been convened and was now ready to give a draft report.

*S Edwards:* Minimum laboratory needs were presented under the headings of 'legal', 'biosecurity', 'equipment' and 'personnel' requirements. A distinction was made between ideal and minimum requirements.

*Discussion:* This mostly concerned biosecurity categorisation and the different systems of designation that exist. It was suggested that although CSFV fits well with the definition given of an OIE Category 3 pathogen, the precautionary measures required for containment are rather stringent. It was concluded that the minimum requirements laid out in the draft should be interpreted as precisely that, with the option for more stringent controls, if so desired, to be superimposed by national governments or local laboratory rules.

*Conclusion:* It was agreed that a draft document would shortly be sent to each of the NSFLs for comment. An updated version of the guidance would be presented at next year's NSFL meeting.

### The draft document is shown in ANNEX I

#### EU Diagnostic manual for CSF

*S. Edwards, K. Depner, F. Koenen, T. Mueller*

*S Edwards:* An outline description of the proposed format of the manual was given. It should be distributed by the CRL. It should comply with the methods recommended by the OIE Manual, but should be more detailed, so as to give full working methods. Two methods were presented, one for the virus neutralisation test, and the other for carrying out FAT on cryostat sections.

*K Depner:* A small panel of sera tested at Hannover, had similar titres of neutralising antibody regardless of whether virus-serum incubation was for one or two hours.

*H de Smit:* Confirmed that this was his experience also.

*K Depner:* Should methods for ASF be included in the manual?

*P Wilkinson:* Good idea, but who would do this?

*J Westergaard:* To discuss idea with S Viscaino.

*J Westergaard:* Idea is to make the manual an official EU document and then to scrap the Annex that currently lays down requirements for diagnostic tests.

*K Depner:* Requested protocols on methods for virus isolation from all NSFLs, so that work could commence on producing a recommended method for the manual.

*S Edwards:* Should RT-PCR be made an officially recognised test by inclusion in the manual?

*V Moennig:* A standardised method is needed. Use of the RT-PCR test should be optional.

*D Rutili:* RT-PCR is a very valuable method, for instance for detection of virus in wild boar samples, and should be available as an option.

*J van Oirschott:* Need to conduct a ring test to ensure satisfactory performance of the test.

*D Rutili:* Should a work-shop be held to compare methods?

*P Lowings:* Considerable practical difficulties in holding RT-PCR workshops because of the need for particular models of specialist equipment. Better to have a ring test in first instance.

*K Depner:* Agreed to liaise with S Belak, concerning preparation of suitable samples for inclusion in the annual ring test for 1998.

*D Paton:* FAIR project group that are working on RT-PCR diagnostic methods will draw up a recommended procedure for presentation and discussion at next NSFL meeting.

### **The draft document is shown in ANNEX II**

#### **Use of Marker Vaccines during CSF epidemics**

*D Paton, V Moennig*

*J Westergaard:* The subgroup has now produced a final document defining a marker vaccine and outlining minimum demands, potential for use and economic aspects. The main conclusions are that the development of such vaccines is feasible; that they offer certain clear advantages over conventional vaccines; but decisions on their use are likely to be controversial and no decision is possible until a final product is available for assessment.

*V Moennig/D Paton:* Outlined needs for further research, as presented in the document.

*H de Smit:* Is it possible to make a preliminary approach to trade partners to canvass their likely views on use of this type of vaccine in Europe?

*J Westergaard:* Informal enquiries have been made, but the first official step will have to be to reach a consensus amongst member states.

#### **Conditions for waste food (swill) feeding pigs**

*J. Westergaard*

##### **1. GENERAL LEGISLATION**

In accordance with tile provisions of Council Directive 80/217/EEC introducing Community measures for the control of classical swine fever „swill“ is defined as: waste from kitchens, restaurants or, as the case may be, from industries using meat.

The responsibility for the implementation of the control measures related to swill feeding rests with the Member States. The legal requirements are given in Artricle 15 of Council Directive 80217/EEC. The text is shown below:

##### Article 15

Member States shall ensure that:

1. the use of swill originating from means of international transport, such as ships, land vehicles or aircraft, is prohibited for the feeding of pigs and that such swill is collected and destroyed under official supervision.
2. swill for the feeding of pigs must be heat-treated so as to ensure the destruction of swine fever virus. Swill so treated may be used for feeding to fattening pigs only and pigs fattened on a holding using such swill may leave the holding only to go for slaughter.

However, the competent authority may allow the feeding of other categories of pigs with swill provided that the pigs kept on the holding cannot leave except to go for slaughter;

3. the collection, transport and treatment of swill intended for feeding to pigs are subject to official authorization.

Swill must be transported in vehicles or containers so designed that it cannot leak or fall out of the vehicle during transport.

Each time after use, the vehicles or containers used for the transport of swill must be cleaned and disinfected according to the instructions of the competent authority;

4. the authorization referred to in paragraph 3 for high treatment of swill is granted subject to the following conditions:
  - the holding must have completely separate facilities for treated and untreated swill,
  - the premises for storage of untreated swill and the premises where treatment takes place must be easy to clean and disinfect;
5. swill collected in accordance with paragraph 3 may be used only on the holding where it has been heat-treated.

Member States may authorize the treatment of swill in specialized establishments equipped for the purpose, on which there are no animals and which are under official control. In this case, by way of derogation from paragraph 2, the swill may, after heat-treatment, also be used for the feeding of pigs other than fattening pigs, provided that its distribution and use are controlled so as to avoid any risk of the swine fever virus spreading;

6. the authorization referred to in paragraph 3 is not required in the case of small holdings using their own swill for feeding to their own pigs, provided that such swill is heat-treated in a manner such as to ensure the destruction of swine fever virus.

## 2. LEGISLATION

In January 1997 Classical Swine Fever was recorded in Kreis Paderborn, Land of Northrein-Westphalia, Germany. The epidemiological investigation carried out revealed that the source of infection could be linked to swill feeding which did not comply with the existing legislation. With the aim to highlight the importance of effective swill feeding controls the Commission included in Commission Decision 97/116/EC of 11 February 1997 temporary requirements as shown below:

### Article 7

1. Germany shall take measures to prevent the spread of classical swine fever through the feeding of waste food (swill) to pigs; the measures shall include:

inspection twice a month of all holdings authorized to treat swill intended for feeding to pigs. The objective of the inspections shall be to verify the compliance with conditions of the issued authorization and the application of sanitary measures to prevent recontamination;

information on the spread of classical swine fever, disease eradication, the potential trade implications and on ways to ensure safe waste disposal. The

information should target pig holders, hunters and owners of restaurants and similar catering facilities.

2. By 1 June 1997 Germany shall present a report to the Commission on the implementation of the campaign referred to under paragraph 1 including at the level of each *Land*:

the number of holdings authorized, in accordance with Article 15(3) of Directive 80/217/EEC, to carry out treatment of swill to be fed to pigs;

the number of restaurants and similar catering facilities from which the collection of waste food (swill) is authorized,

the findings and actions taken in relation to inspections carried out.

### 3. SWILL FEEDING IN MEMBER STATES

In relation to the adoption of Commission Decision 97/116/EC the Commission invited all Member States to provide information on:

- (a) holding authorized to carry out treatment of swill to be fed to pigs;
- (b) restaurants, etc. authorized to supply waste food for treatment;
- (c) inspections carried out.

Information related to the points (a) and (b) is given in the attached tables.

**Table 1:** Information on feeding of swill to pigs

MEMBER STATE	HOLDINGS*	RESTAURANTS**
Belgique/Belgie		
Danmark	1	
Deutschland	407	32.109
Ellas	none	
Espana		
France		
Ireland		
Italia		
Luxembourg		
Nederland		
Österreich	236	
Portugal		
Finland - Suomi	60	
Sverige	140	
United Kingdom	180	7.258

\* Holdings authorized to feed swill to pigs.

\*\* Restaurants and other catering facilities authorized to supply waste.

**Table 2: Information on inspections carried out on holdings authorized to feed swill to pigs.**

MEMBER STATES	INSPECTIONS
Belgique/Belgie	
Danmark	
Deutschland	
Ellas	
Espana	
France	
Ireland	
Italia	
Luxembourg	None. Total ban on swill feeding
Nederland	
Österreich	
Portugal	
Finland - Suomi	
Sverige	
United Kingdom	At least 4 inspections per year

## **FUTURE WORK, CONCLUSIONS AND RECOMMENDATIONS**

### **Proposal for the work of the Community Reference Laboratory for Classical Swine Fever**

#### **I. Contractual duties.**

The functions and duties are specified in Annex VI of Council Directive 80/217/EEC (Official Journal of the European Communities No L 166 of 8.7.1993).

#### **II. Objectives for the period October 1997 - September 1998.**

1. Collecting and editing of material for a report covering the annual meeting of National Swine Fever Laboratories held in Vienna, Austria.
2. Prepare programme and working documents for Annual Meeting of National Swine Fever Laboratories, 1998.
3. Prepare programme and working documents for Annual Meeting of National Swine Fever Laboratories from Eastern and Central Europe.
4. Selection and inoculation of pigs for the production of CSF sera for the inter-laboratory comparison test to be carried out by the National Laboratories.
5. Quality control and distribution of the reagents prepared to carry out the inter-laboratory comparison test.
6. Analysis of results submitted by Member States as regards the inter-laboratory comparison test.
7. Preparing a manual containing detailed protocols for laboratory tests for the diagnosis of CSF.
8. Re-stocking of CSF video and preparation of video in Swedish and Finish.
9. Perform check on stock of cell cultures, strains of virus and selected reagents.
10. Examine new isolates of CSF virus submitted by NSFL and build up virus collection.
11. Evaluation of data from Member States on molecular epidemiology of CSF for the purpose of the CRL. Support on ongoing epidemiological research projects on recent outbreaks of CSF.
12. Biological characterization of recent isolates of CSF virus
13. Transmission experiments of CSF virus via contaminated semen (clinical, virological and serological investigations).
14. Analysing the CSF situation in the wild boar population in Europe (e.g. preparation of a meeting)

## **Conclusions and Recommendations**

The CSF and ASF situation in the Community and the progress made in research, diagnostic procedures, epidemiology and disease control were reviewed. After this, the participants of the meeting defined a number of recommendations considered to be of importance for the eradication of CSF and ASF from the Community and the work to be performed by the NSFLs. The recommendations proposed are listed below.

### **Swill feeding**

The feeding of waste food to pigs has continued to cause outbreaks in a number of Member States. It is therefore recommended that:

- existing legislation to control waste food processing and feeding must be strictly enforced,
- border controls (third countries) should be tightened,
- public awareness concerning dangers of swill feeding should be increased (hunters, farmers, restaurant owners, tourists etc.),
- higher hygienic standards in the farming industry (motivation) must be propagated,
- the processing of waste food must not take place on or near premises where pigs are kept.

### **Continuation of work on standardization of diagnostic methods**

The draft version of the manual containing detailed protocols for laboratory tests for the diagnosis of CSF should be updated and complemented with protocols for PCR, NIF test and virus isolation methods. The updated version should be presented at the next annual meeting of the NSFLs.

### **Standardization of pestivirus nomenclature**

In the sense that isolates and strains can easily be identified.

### **Continuation and completion of the CSFV strain collection at the CRL in Hannover**

Data should be made available to authorized laboratories through the internet.

### **Improvement of control of CSF in wild boar**

Preparation of a meeting to collect all related knowledge and to reactivate the existing subgroup.

### **Laboratory diagnosis of ASF**

Although the ASF situation in Europe is encouraging (as shown by Dr. Patta) the diagnostic capabilities of Member States should be conserved by organizing and performing an interlaboratory test.

## ANNEX I: MINIMUM REQUIREMENTS FOR NATIONAL SWINE FEVER LABORATORIES

Report of an expert group, 18 March 1997

Those present: K Depner (EU and OIE Reference laboratory, Hannover, Germany)

*Chairman*

S Edwards (OIE Reference laboratory and NSFL, Weybridge, UK)

*Rapporteur*

F Koenen (NSFL Brussels, Belgium)

T Müller (NSFL Wusterhausen, Germany)

Commission representative: J Westergaard

### Agenda

1. Introduction
2. Review minimum biosafety requirements for national swine fever laboratories (NSFL)
3. Review minimum requirements for the equipment and personnel of NSFL

#### **1. Introduction**

The group met to consider recommendations 3, 4, and 5 agreed at the Annual Meeting of NSFLs in Alghero, Sardinia in June 1996 [1].

The NSFL in each Member State should be self-sufficient in equipment and facilities at such a level that it can fulfil the requirements of Annex I of Directive 80/217. Permits for the laboratory to hold classical swine fever virus (CSFV), with any controls or limitations on its usage, must comply with national and community legislation.

*Note:* in the following text the term „unit“ refers to the dedicated room or rooms which are specified within an institute for carrying out the functions of NSFL.

#### **2. Minimum biosafety requirements for national swine fever laboratories**

It was agreed that the biosecurity arrangements for NSFL should be defined before deciding on the other requirements needed to carry out this function.

Classical swine fever virus (CSFV) is not a significant hazard for human health. Biosecurity arrangements should therefore be designed to prevent escape of infectious virus from the laboratory to susceptible pig populations. A number of general texts are available on biosecurity measures [2, 3] however the principle source in this context is the draft OIE text [4] on „International Transfer and Laboratory Containment of Animal Pathogens“ (*OIE International Animal Health Code, proposed chapter 1.5.6*).

The group agreed that CSFV falls into the OIE „Group 3“ of animal pathogens by the majority of criteria. This is defined as *disease producing organisms which are either exotic or which are enzootic but subject to official control, and which have a moderate risk of spread from the laboratory*. Bearing in mind the characteristics of CSF virus, it is considered that full containment level 3 may not be required under all circumstances, and local risk assessments should be carried out for specific situations. In doing so, the nature of the activities undertaken by a particular laboratory should be taken into account. Post mortem examinations, processing of tissues for cryostat sections, and serology using inactivated antigen, are relatively low risk procedures which could be carried out at a lower containment level involving basic hygiene and post-operational disinfection/decontamination with safe disposal of tissues and sera. These conditions might obtain for example in a regional laboratory. In contrast, procedures involving multiplication of the virus (virus isolation in cell culture, growth of known virus stocks in cell

culture, virus neutralisation tests) should be contained at level 3. This is the standard to which all NSFL should aspire. It is possible to define „minimum“ containment criteria for CSFV, while in parallel identifying „ideal“ containment conditions which would be regarded as desirable for laboratories cultivating large stocks of virus (Table 1). Any suspect clinical or pathological case may be either classical or African swine fever. The facilities suggested for CSFV should equally be applicable to ASFV.

**Table 1: Principles of biological containment appropriate for CSFV laboratories**

	<b>Ideal requirement</b>	<b>Permitted interim arrangement</b>
General Environment	Negative pressure controlled ventilation. Double HEPA filtration of exhaust air. Dedicated rooms, used exclusively for swine fever diagnostic procedures.	Normal atmospheric pressure.  Dedicated rooms limited to defined procedures.
Laboratory clothing.	Complete change of clothes on entry. Laboratory clothing used only in the CSFV unit. Disposable gloves for all manipulations of infected material. Clothing sterilised before removal from unit, or washed within unit.	Dedicated outer clothing used only in the CSFV unit. Disposable gloves for all manipulations of infected material. Outer clothing sterilised before removal from unit, or washed within unit.
Control of personnel	Entry to unit limited to named, trained personnel. Full shower on exit from unit. Personnel not permitted near pigs for 48 hours after leaving unit.	Entry to unit limited to named, trained personnel. Wash hands on leaving unit. Personnel not permitted near pigs for 48 hours after leaving unit.
Equipment	Biological safety cabinet (Class I or II) used for all manipulations of live virus. Cabinet should have double HEPA filtration of exhaust air. All equipment needed for laboratory procedures to be available within the dedicated laboratory suite.	

In addition to the above measures, all stocks of CSF virus must be kept in secure storage, whether deep frozen or freeze dried. Freezers and refrigerators should not be used for viruses other than CSFV, or for other materials unrelated to CSF diagnosis. All individual ampoules must be clearly labelled, and comprehensive records maintained of virus stocks together with dates and results of quality control checks. Records must also be kept of viruses added to stock, with details of the source, and of viruses issued to other laboratories.

The biosecure unit for CSFV work should be supported by „clean“ areas for the preparation of glassware and media, the maintenance and preparation of noninfected cell cultures, the processing of sera and serological testing (other than methods using live CSFV), and the provision of administrative and clerical support.

Any work with CSFV involving experimentally infected animals presents a high risk of spread of the virus, and must be carried out under adequate containment (Table 2).

Table 2: Principles of biological containment for CSFV experimental animal rooms

	Obligatory requirements
General Environment	Negative pressure controlled ventilation. Double HEPA filtration of exhaust air. Facility for complete fumigation/disinfection at end of experiment. All effluents treated to inactivate CSFV (heat or chemical).
Laboratory clothing.	Complete change of clothes on entry. Disposable gloves for all manipulations. Clothing sterilised before removal from unit, or washed within unit.
Control of personnel	Entry to unit limited to named, trained personnel. Full shower on exit from unit. Personnel not permitted near pigs for 48 hours after leaving unit.
Equipment	All equipment required for animal procedures to be available within the unit. All materials to be sterilised on removal from unit or, in the case of animal samples, to be double wrapped in leakproof container which is surface disinfected for transport to the CSFV laboratory.
Animals	All animals to be slaughtered before leaving the unit, post mortem examinations to be completed within the biosecure area, and carcasses incinerated on completion of examinations.

### 3. Minimum requirements for the equipment and personnel of NSFL

#### 3.1 Accommodation

A fully equipped unit should contain the following facilities:

- Post mortem room with sink for handwashing and facilities for cleansing and disinfection.  
*Note:* in some cases this facility may be at a remote location, for example in a regional laboratory. In such circumstances arrangements must be in place for the safe and rapid transport of tissue samples to the diagnostic laboratory.
- Biosecure laboratory as detailed above, for work with diagnostic samples and other procedures involving live CSFV. The unit should be strictly separated from other laboratories, especially those dealing with cattle, sheep or other pig diseases to avoid cross-contamination (particularly with BVD virus).
- Changing room with showers and/or handwash basin at the entry/exit point to the biosecure laboratory.
- Secure plant room for refrigerators, freezers, liquid nitrogen tanks.
- Microscopy room, including blackout facility for fluorescence work.
- Clean room(s) for preparation and maintenance of cell cultures, media, and glassware.
- Serology room for tests using inactivated antigens (ELISA, IFA).
- Office accommodation appropriate to the number of staff.
- Additional rooms will be required for any laboratory carrying out PCR or other molecular techniques. The requirements needed for such a facility should be ascertained from experts before embarking on these techniques.

#### 3.2 Equipment

The number of each item of equipment, and the capacity of individual items, will depend on the likely sample throughput in the event of an outbreak. As such it should be related to size of the target pig population. There should also be contingency plans for continuing diagnostic work in the event of equipment failure. The following equipment is essential:

- -70°C freezer or liquid nitrogen tank
- -20°C freezer (optionally -40°C)
- +4°C refrigerator, or walk-in cold room

- Cryostat microtome
- Laminar flow safety cabinet, Class II, with filtered exhaust
- Homogeniser or tissue grinding apparatus
- Incubator (preferably CO<sub>2</sub>)
- Inverted light microscope
- Epifluorescence microscope
- Centrifuge
- Water purifier
- Autoclave or sterilisation oven
- Washing machine (if clothes are washed within the unit)
- General laboratory equipment (balance, pH meter, mixer, shaker, water bath, pipettes including multichannel, pipette aids, etc)

For laboratories carrying out serology by ELISA, the appropriate equipment is needed (diluting and dispensing equipment, plate washer, plate reader, computer and software, printer).

For laboratories carrying out PCR-based diagnostic techniques a thermal cycler is necessary, together with apparatus for gel electrophoresis and a system for reading the gels.

Additional equipment, which is useful for advanced studies but non-essential for the diagnostic laboratory, includes a freeze drier, an ultracentrifuge, and apparatus for DNA sequencing.

### 3.3 *Personnel*

One person should be nominated as the head of the NSFL facility, and should be responsible for ensuring that all aspects of biosecurity, equipment provision and maintenance, and personnel are maintained.

The NSFL requires a core of fully trained staff who are familiar with laboratory procedures and the interpretation of diagnostic tests for CSFV. This should comprise a minimum of one veterinarian and three technical scientific staff. In addition access should be available to a wider group of veterinary and scientific staff, normally assigned to other duties, who have sufficient training to assist in the event of a disease emergency and can also provide cover for unavoidable absences of the core staff.

Training records should be maintained and kept up to date for all staff of the unit, including details of the tests and procedures for which they are fully trained, and others which they may carry out under supervision.

Although the NSFL is not directly responsible for field veterinary staff, it has a responsibility to assist the national veterinary authority with training of field staff to ensure that they are familiar with the signs and lesions of CSF and are aware of the statutory procedures to be followed in the event of a suspected outbreak.

## ANNEX II: EU DIAGNOSTIC MANUAL FOR CSF

### The virus-neutralization test (VNT)

Method written: 1997

Last update: 30.5.97

Update No: 2

The detection of neutralizing antibodies against classical swine fever (CSF) virus in serum samples is carried out to assist in the diagnosis of CSF in holdings containing pigs showing clinical signs of the disease or in pigs believed to have had contact with infected pigs. It may also be carried out for the purpose of surveillance or for surveys in herds of unknown status as well as for epidemiological investigations.

#### Principle of test

The test is carried out in flat-bottomed microtitre plates and is based on the determination of the neutralizing 50% endpoint. Therefore a constant amount of CSF virus of 100 (plus/minus 0.5 log<sub>10</sub>) virus infectious doses (TCID<sub>50</sub>) is incubated with diluted serum for one hour at 37 °C. For screening purposes, the sera are initially diluted 1/5. When a full titration is necessary two-fold dilutions of serum starting at 1/2 or 1/5 are prepared. At least two wells of PK-15 cell cultures are inoculated with mixtures of virus and diluted serum at each dilution. The plates are incubated at 37 °C for 3 to 4 days. After this incubation period the cell cultures are fixed and the viral antigen is detected by an immune labelling system. Either the neutralization peroxidase-linked antibody (NPLA) or the neutralization-immunofluorescence (NIF) assays may be used. The results are expressed as the reciprocal of the initial serum dilution at which half the inoculated cell cultures fail to show any specific labelling (no viral replication detectable in the cell culture). A point between two dilution levels is estimated.

The VNT is regarded as the most sensitive test to detect antibodies against CSF virus. The peroxidase system has the advantage that the results can be read with the light microscope or even with the naked eye. For the NIF test a fluorescence microscope is needed.

#### CSF virus strain for the VNT

##### *a) Stock virus)*

The reference strain of virus for use in the European Union is derived from infectious cDNA of the CSF virus strain Alfort/187 (Ruggli, N.; Tratschin, J. D.; Mittelholzer, C., and Hofmann, M. A., 1996. *Journal of Virology* 70: 3478-3487). The reference virus can be supplied by the Community Reference Laboratory for CSF, upon request.

Batches of stock virus are produced by inoculating one day old PK-15 cell cultures in tissue culture flasks with 0.5ml of the reference virus. The cultures are incubated for 3 to 4 days at 37 °C and thereafter frozen at -80 °C. After thawing, the culture fluids are clarified by centrifugation for 60 minutes at 3000g. The supernatants are dispensed in 1ml amounts in ampoules which are labeled, packed and stored at -80 °C until use in the VNT.

##### *b) Virus titration back-titration*

Three randomly selected ampoules are titrated from each batch of stock virus. Serial ten-fold dilutions of virus (e.g. 0.9ml medium + 0.1ml virus suspension) are prepared in test tubes from each ampoule. Stock virus is titrated from 10<sup>-1</sup> to 10<sup>-8</sup>. A fresh pipette must be used for each serial dilution step. The diluent consists of cell culture medium. Virus suspensions from the test tubes are then transferred to a flat-bottomed microtitre plate in amounts of 50µl per well and

four wells per dilution of virus. Wells containing medium only, are inoculated in each titration for control. 100µl PK-15 cell suspension (100 000 cells/ml) is added to each well and the plate incubated for 3 to 4 days at 37 °C in a CO<sub>2</sub> incubator. The cultures are fixed, stained with peroxidase or fluorescent conjugate and finally examined for viral antigen.

A back-titration must be mounted on every occasion that a VNT is carried out. It is carried out using the actual virus added to the VNT plate, and covers a range of 4 log dilutions (ie 10<sup>0</sup> to 10<sup>-3</sup>). The back titration thus acts as one of the internal quality controls. If the back titration is outside the tolerance limits (30-300 TCID<sub>50</sub> per well) then the test is invalid and must be repeated.

#### *c) Calculation of infectivity titres*

The highest dilution of virus which infected 50% of the cell cultures is regarded as the endpoint of infectivity. This value is estimated or calculated using the method of KÄRBER (1931). Virus infectivity titres are expressed as tissue culture doses (TCID<sub>50</sub>) per volume (e.g. 0.1ml) of virus suspension.

**Example:** All wells containing virus dilutions from 10<sup>-1</sup> to 10<sup>-5</sup> are antigen positive. Two of four wells containing the virus dilution of 10<sup>-6</sup> are antigen positive. The remaining wells containing higher virus dilutions are negative. In this case the titre is 10<sup>6</sup> TCID<sub>50</sub>/volume. If only one out of four wells from the dilution of 10<sup>-6</sup> is positive the titre would be estimated as 10<sup>5.7</sup>.

#### **Cell culture for VNT**

PK-15 cells proven to be free of pestivirus contamination are used for the VNT. The cells can be supplied by the Community Reference Laboratory for CSF, upon request.

For the passage of the PK-15 cells the culture medium is decanted and the cell monolayer thereafter rinsed once with 10ml prewarmed PBS-V. The flask is replenished with 10ml fresh PBS-V and incubated for 10 minutes at 37 °C. After discarding the PBS-V solution the flask is renewed with 10ml PBS-V containing 0.1ml of 1% trypsin solution followed by re-incubation at 37 °C for 10-20 minutes. At periodic intervals, the flask is microscopically controlled for evidence of cell detachment. Following complete disintegration of the cell monolayer, the cell suspension is decanted into a centrifuge tube containing 2ml culture medium. After vigorous mixing by pipetting up and down, the cell suspension is centrifuged at 600-800g for 10 minutes. The cell pellet is reconstituted in 10ml culture medium and thereafter the number of suspended cells is determined by counting in a cell counting chamber (e.g. Thoma chamber). Finally the cell suspension is diluted with culture medium to give concentrations of 100 000 - 150 000 cells/ml for subculturing in cell flasks or microtitre plates.

#### **Test procedure**

##### **Neutralization peroxidase-linked antibody assay (NPLA)**

##### *a) Neutralization reaction*

1. Load 80µl cell culture medium plus 20µl serum sample in the first row of wells of the microtitre plate to obtain the initial serum dilution of 1/5. Two wells per serum dilution are used. The remaining wells of the plate are loaded with 50µl of medium. Thereafter, 50µl of the 1/5 serum dilution are withdrawn by means of a 12 channel pipette and diluted serially two-fold. (When titration is finished each well contains 50 µl of serum-medium dilution.)
2. Add 50µl/well of test virus suspension containing 100 TCID<sub>50</sub>/50µl and gently shake the plate. The required dilution of the test virus to obtain 100 TCID<sub>50</sub>/50µl has to be prepared shortly before use by diluting the virus in growth medium.

3. Place the plates in a moist chamber and incubate in a CO<sub>2</sub> incubator (4-5% CO<sub>2</sub>) for 1 hour at 37 °C. (Alternatively the plates can be sealed air tight and incubated for 1 hour at 37 °C)
4. Add 100µl/well of growth medium containing approximately 100 000 cells/ml and shake gently for 5-10 seconds. The cell suspension should be prepared during the 1 hour incubation period.
5. Place the plates in a moist chamber and incubate in a CO<sub>2</sub> incubator (4-5% CO<sub>2</sub>) for 3-4 days at 37 °C. (Alternatively the plates can be sealed air tight and incubated for 3-4 days at 37 °C)
6. Discard the growth medium and rinse the plates once in 1/3 PBS (1/3 PBS + 2/3 distilled water). Drain the plates by blotting on paper towels.
7. Fix the cell monolayers by one of the following methods:
  - incubate the plates at 70-80 °C for 2 hours
  - add 100µl/well of acetone/methanol (1:1) solution for 10 minutes at room temperature
  - fix in 20% acetone for 10 minutes. Drain plates thoroughly then dry under a bench lamp for 4 hours at 25-30°C
 (Fixed plates may be stored before staining for several days at 4 °C)

*b) Immune labelling*

1. Rinse the plates once in 1/3 PBS
2. Add to each well 50µl working dilution of a pestivirus conjugate and incubate for 1 hour at 37 °C.
 

*Alternative method for indirect labelling:*

  - a) Add to each well 50µl working dilution of a pestivirus specific antiserum or monoclonal and incubate for 15 minutes at 37 °C.
  - b) Wash as in step 3 below.
  - c) Add to each well 50µl working dilution of an antispecies peroxidase conjugate and incubate for 15 minutes at 37 °C. Continue at step 3.
3. Wash the plates 3 times with PBS-Tween and one time with distilled water. Drain the plates by blotting on paper towels.
4. Add to each well 50µl of chromogen-substrate solution and stain for 15-30 minutes at room temperature.
5. Discard the chromogen-substrate solution and add 100µl/well of 1/3 PBS. Read the test by low-power microscopy. The cytoplasm of infected cells is stained dark red.

*c) Controls*

Back-titration of test virus must be carried out each time to check if the virus titre was 100 TCID<sub>50</sub>/50µl (tolerance of 0.5 log either way: 30-300 TCID<sub>50</sub>/50µl). Reference negative and positive serum samples as well as cell controls containing medium and cells have to be included in the test. The test has to be repeated if the controls do not correspond with the expectations. Reference sera can be supplied by the Community Reference Laboratory for CSF and by the OIE Reference Laboratories for CSF, upon request.

*d) Estimation of ND<sub>50</sub>*

Serum titres are recorded as reciprocal of the highest **initial dilution** of sera (dilution of serum and growth medium without virus suspension) which prevented virus replication in 50% of the wells. Wells are scored as virus positive even if only one cell within the monolayer stains specifically. A point between two dilution levels is estimated. Serum titres are expressed as neutralization dilution 50% (ND<sub>50</sub>) and can be also calculated using the method of KÄRBER (1931).

Example 1: Only one of the two wells of the serum dilution of 1:10 has infected cells. In this case the neutralization titre would be 10 ND<sub>50</sub>.

Example 2: All wells up to the dilution of 1/80 are free of viral antigen while the remaining wells with serum dilutions equal and higher than 1/160 are positive. In this case the neutralization titre is estimated to be 120 ND<sub>50</sub>.

When reporting test results, especially for export tests to other countries, it is important to specify clearly that the titre is expressed as Initial Dilution, and that for Final Dilution (which is the method mostly used in America) the result should be multiplied by 2 (*ie* 1/10 initial, corresponds to 1/20 final). The final dilution system is based on the actual dilution of serum during the neutralization reaction, *ie* after addition of virus but before adding the cell suspension.

**Calculation of dilution factor for obtaining 10<sup>2</sup> TCID<sub>50</sub>/50µl**

As an example consider the stock virus has a titre of 10<sup>5.7</sup> TCID<sub>50</sub>/100µl. The virus dilution employed in the VNT should have a titre of 100 (10<sup>2</sup>) TCID<sub>50</sub>/50µl.

10<sup>5.7</sup> TCID<sub>50</sub>/100µl correspond to 10<sup>5.4</sup> TCID<sub>50</sub>/50µl

*calculation:*

10<sup>5.7</sup> TCID<sub>50</sub>/100µl is to be divided by 2 (2 is equivalent to 10<sup>0.3</sup>) - that means:  
10<sup>5.7</sup> divided by 10<sup>0.3</sup> - that means: 5.7 - 0.3 = 5.4 (10<sup>5.4</sup>)

10<sup>5.4</sup> TCID<sub>50</sub>/50µl are to be diluted to obtain 10<sup>2</sup> TCID<sub>50</sub>/50µl

*calculation of dilution factor:*

10<sup>5.4</sup> is to be divided by 10<sup>2</sup> = 10<sup>3.4</sup> (5.4 - 2 = 3.4)

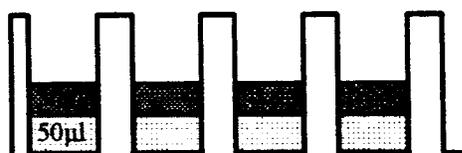
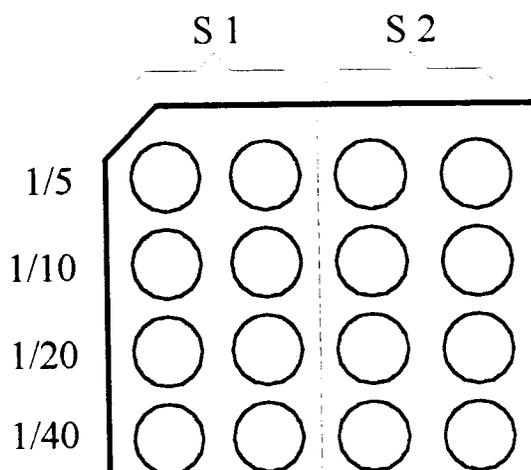
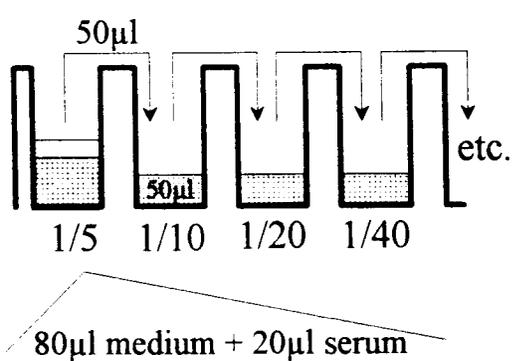
**dilution factor = 1/10<sup>3.4</sup> - that means: approx. 1/2600**

(From 1ml stock virus containing 10<sup>5.7</sup> TCID<sub>50</sub>/100µl (or 10<sup>5.4</sup> TCID<sub>50</sub>/50µl) one can make 2600ml of virus solution containing 10<sup>2</sup> TCID<sub>50</sub>/50µl)

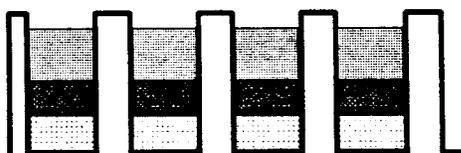
## NPLA

### a) neutralization

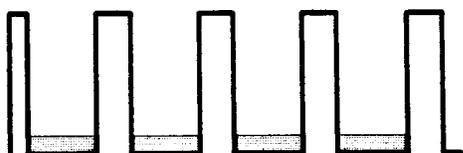
1. Titration of serum in growth medium,  
starting with 1/5,  
2 wells/serum dilution



2. Add 50µl test virus/well,  
incubate 1h at 37 °C



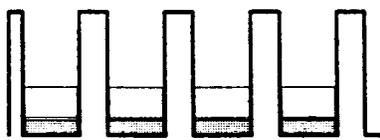
3. Add 100µl cell culture/well,  
incubate 3-4 days at 37 °C, CO<sub>2</sub>



4. Discard medium, rinse once  
in 1/3 PBS, fix plate  
(e.g. 2h at 80 °C)

## NPLA

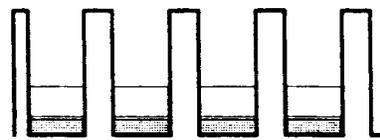
### b) immune labelling



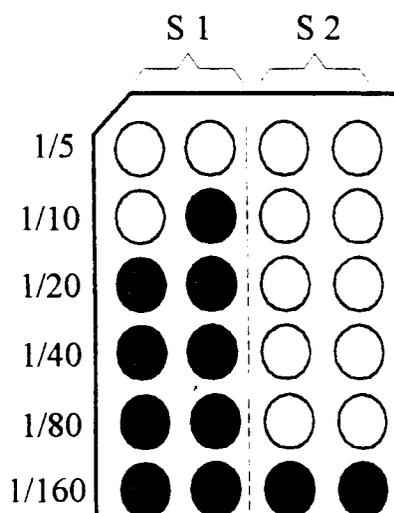
5. Rinse once in 1/3 PBS, add 50  $\mu$ l/well of conjugate and incubate for 1h at 37 °C



6. Wash plate 3 x with PBS-tween and 1 x with distilled water, add 50 $\mu$ l/well chromogen-substrate, incubate 15-30 minutes at RT



7. Discard chromogen-substrate, add 1/3 PBS, read the test by low power microscopy



neutralization titre (NT<sub>50</sub>)

S 1: 10

S 2: 120

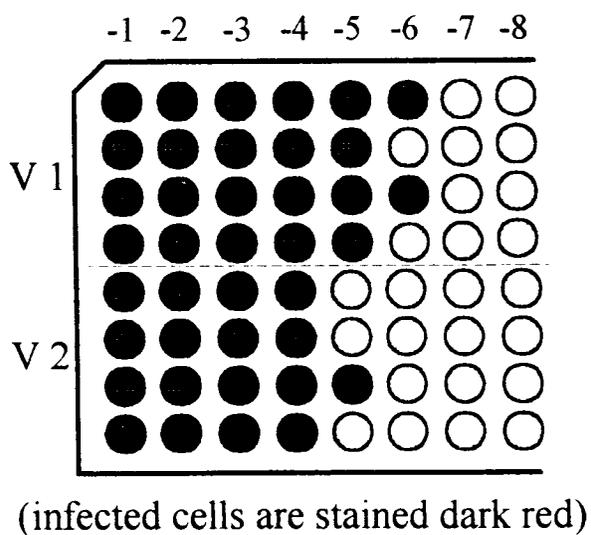
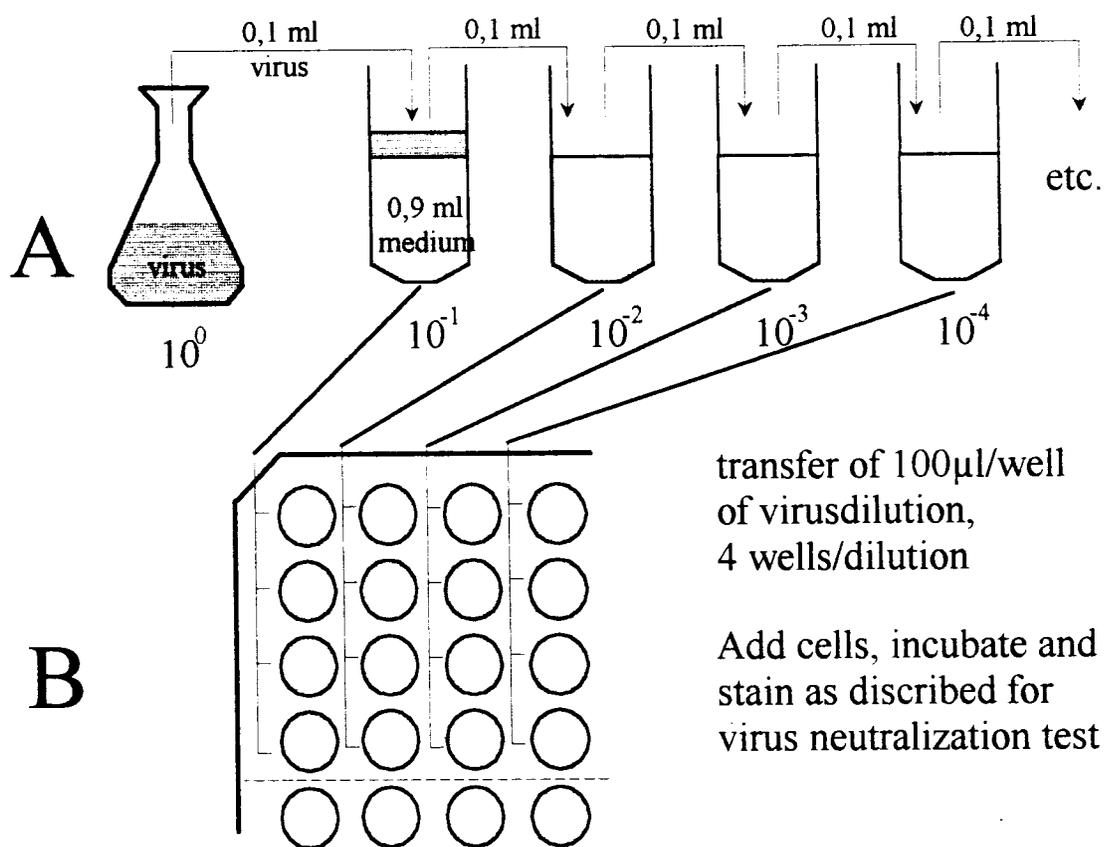
NT<sub>50</sub>: reciprocal of the highest initial dilution of the sera which prevented virus replication in 50% of the wells.

A point between two dilution levels is estimated.

(infected cells are stained dark red)

## Virus titration

log 10 virus dilution



Virus titre (TCID<sub>50</sub>):

V 1:  $10^6$  /0,1 ml  
V 2:  $10^{6,7}$  /0,1 ml

TCID<sub>50</sub>: reciprocal of the initial virus dilution at which 50% of the wells showed virus replication. A point between two dilution levels is estimated.

## **Demonstration of viral antigen in cryostat sections** (Direct Fluorescent Antibody Test - FAT)

### Principle of test

Classical swine fever virus (CSFV) antigen is demonstrated in thin cryostat sections of organ material. The cryostat sections are mounted on a microscope slide and stained directly with anti-CSF immunoglobulin conjugated to fluorescein isothiocyanate (FITC-conjugate). The sections are examined for fluorescence by UV microscopy. The IFT is rapid and reliable.

### Material

Tonsils and spleen tissues are considered essential. Preferably at least two other lymphatic tissues should be collected, such as the retropharyngeal, parotid, mandibular or mesenteric lymph nodes together with distal part of ileum, parotid gland or kidney. Each sample of the tissue should be placed in a separate sealed plastic bag and labelled. The samples should be transported without preservatives at refrigerated temperature (not frozen!) in leak-proof containers and tested without delay.

### Test procedure of direct FAT

1. Cut out a piece of tissue of approx 1 x 1 x 0,5 cm and mount it with a cryo-embedding compound or distilled water on a cryostat mounting block (chuck).
2. Freeze the piece of organ onto the cryostat chuck. The freezing temperature of the cryostat should be -15 °C.
3. Cut sections of maximum 5 µm thickness and mount them onto microscope slides which have been previously cleaned with alcohol. Prepare at least three slides with sections from the same tissue. Alternatively the sections can be picked up on a coverslip.
4. Dry the mounted sections at room temperature for 20 minutes.
5. Fix the mounted sections for 10 minutes at room temperature in acetone (analytic grade).
6. Immerse the section briefly in washing buffer, remove excess fluid with tissue paper and place them on a frame in a humid incubation chamber with a little water in the bottom to reduce evaporation.
7. Remove a fixed control positive section from the deep freeze (-70 °C) and process in parallel.
8. Dispense the FITC-conjugate at working dilution onto the entire section. close the moist chamber and incubate in the dark for 30 minutes at 37 °C (make fresh working dilutions of conjugate with washing buffer for each series of organ samples).
9. Wash the sections 3 x 10 minutes at room temperature with washing buffer;
10. Immerse the section briefly in distilled water.

11. Carefully remove excess fluid with tissue paper and place a cover slip with mounting buffer onto the section (section between cover slip and slide).
12. Remove excess mounting fluid with tissue paper and examine the sections for fluorescence by UV microscope.

### Controls

Negative and positive control sections must be included in each series of organ samples to be examined. The control sections can be prepared in advance and stored after acetone fixation for several months at -70 °C.

### Interpretation

Any sample showing specific cytoplasmic reaction (brilliant green fluorescence) shall be considered positive for pestivirus. In such cases the FAT positive result should be confirmed by virus isolation on cell culture. Cryostat sections alone should not be used as the sole diagnostic method in a country or region which is free of CSF. The purpose of carrying out cryostat testing is to provide a rapid result. The method is also useful for screening large numbers of samples in the face of an outbreak.

A negative FAT result does not necessarily rule out CSF in all cases. In some cases during the terminal stage of the disease a positive reaction can be masked by neutralizing antibodies which are already induced by the organism. When suspicion of CSF continues further samples should be examined. In subacute and chronic cases, the ileum is frequently positive and occasionally may be the only tissue to display fluorescence.

Pigs vaccinated with modified live virus strains may yield a positive FAT result for two weeks after vaccination. Strains of modified live virus vaccine multiply mainly in the regional lymph nodes and in the crypt epithelium of the tonsils. Depending on the type of vaccine it may be possible to carry out differential staining with vaccine-specific monoclonal antibodies.

Pigs infected with bovine viral diarrhoea (BVD) or border disease (BD) viruses can give false positive results when a FITC-conjugate prepared from a polyclonal antibody specific for pestiviruses has been used. For this reason it is recommended that, especially in CSFV-free areas, duplicate samples should be examined from FAT positive cases using monoclonal antibodies which can distinguish CSF virus from BVD or BD viruses. Alternatively, confirmatory diagnosis should await virus isolation in cell culture with subsequent typing by monoclonal antibodies (see method \*\*\*\*\* - Typing of pestiviruses with monoclonal antibodies).

### FITC-conjugate

The quality of the FITC-conjugate determines the quality of the reaction. It is recommended that primary diagnosis is carried out with FITC-conjugates prepared from a polyclonal antibody to CSF. This will not distinguish between the antigens of different pestiviruses, but does provide assurance that minor variant viruses will not be missed. FITC-conjugates should be prepared from hyperimmune serum prepared in specific pathogen free pigs. The serum should be free from any antibody which could affect the specificity or quality of the specific CSF reaction.

The conjugate should have a minimum working titre of 1/20 as determined in CSF virus-infected cell cultures and confirmed by check tests on tissue sections. The working dilution of the conjugate should combine a maximum of signal with a minimum of background staining.

#### ANNEX

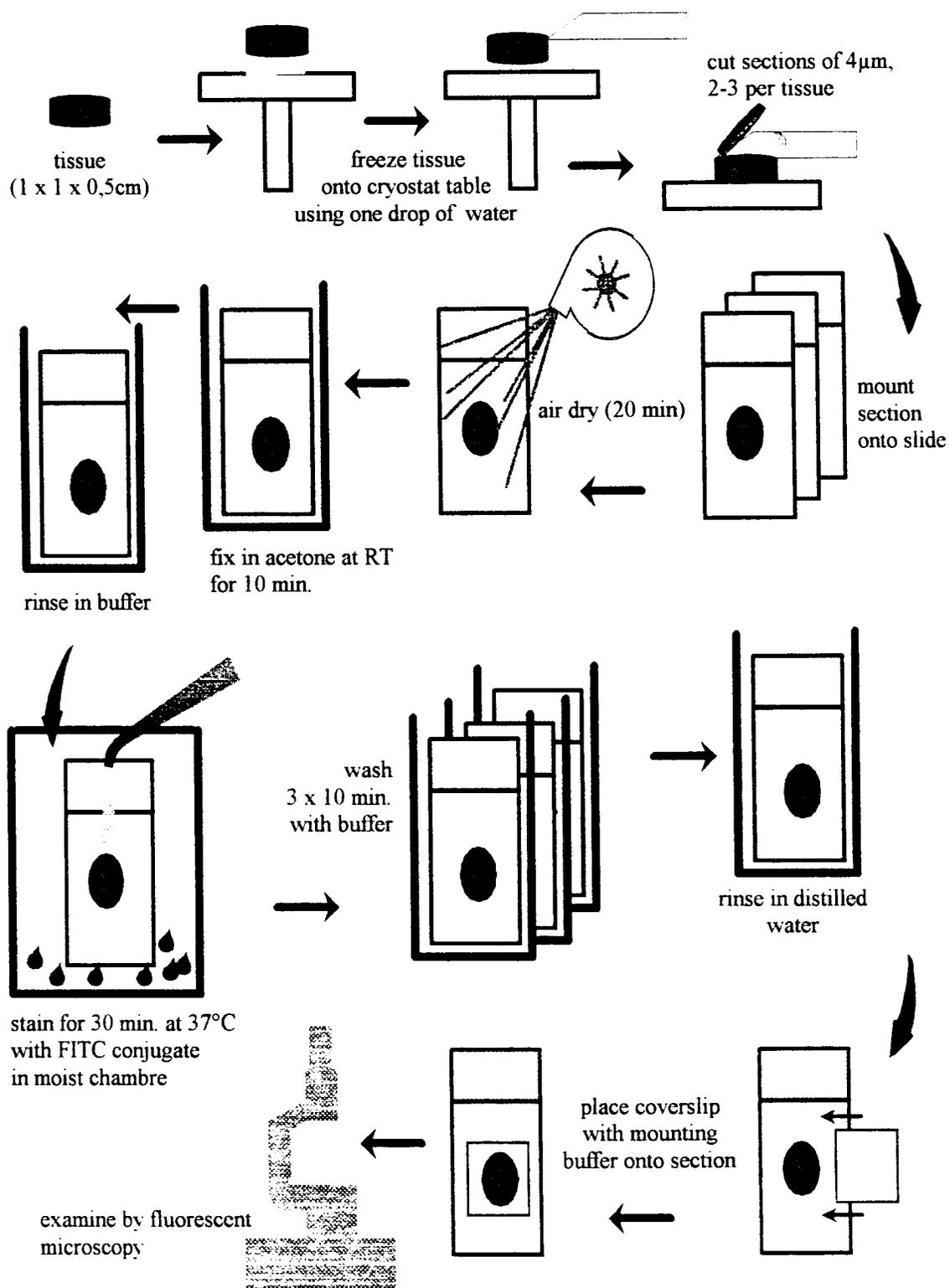
washing buffer: Phosphate buffered saline (pH 7.4 - 7.6):  
(Physiological saline buffered with 0.01M phosphate)  
NaCl 8.78 g/l  
Na<sub>2</sub>HPO<sub>4</sub> x 12H<sub>2</sub>O 3.58 g/l  
(pH adjusted with 1M KH<sub>2</sub>HPO<sub>4</sub>)

#### mounting buffer

washing buffer with 20% glycerine (buffered glycerine)

*Or we recommend commercial NON-FADING mountant.*

Demonstration of viral antigen in cryostat sections  
(Direct Fluorescent Antibody Test - FAT)



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**ANNEX IV: EPIDEMIOLOGY AND CONTROL OF CSF IN THE CZECH  
REPUBLIC**

# Epidemiology and control of CSF in the Czech Republic

J.Holejšovský

## I. Epidemiology of CSF

Since the early seventies the CSF had not been detected in Czech Republic neither in wild boar nor in domestic pigs until October 1990 when the first outbreak occurred in wild boar in the locality Valtice district Břeclav. This first case after such a long time was preceded by several outbreaks in neighbouring districts (Mistelbach and Holabrun) in Austria during the spring and summer of the same year.

The following year (1991) the CSF spread among the wild boar in the districts Brno-venkov, Třebíč, Znojmo and Žďár nad Sázavou and the first outbreak in domestic pigs in a small farm (less than 20 pigs) in district Brno-venkov after swill (wild boar) feeding occurred.

In the year 1992 CSF continued to spread in the other districts (Jihlava, Pelhřimov, Jindřichův Hradec) keeping the north-western direction. In January of this year the second outbreak in small domestic pigs farm occurred. The origin of this case was the same like in the first case - swill feeding after the wild boar was consumption in the family of the farmer.

In the year 1993 the CSF occurred in wild boar only in the districts Jihlava, Jindřichův Hradec and Pelhřimov, e.g. in the districts of Bohemia-Moravia highlands. In December of this year the only case in wild boar occurred in the locality Turovec in the district Tábor.

In the year 1994 two outbreaks only were recorded in wild boar and one outbreak in the locality Turovec in the big domestic pig farm with about 6 000 pigs. Since July 1994 no outbreak was recorded neither in wild boar nor in domestic pigs.

In the year 1995 no outbreak of CSF occurred until the end of June and the Czech Republic had been 12 months free of CSF. At the end of June there was a new outbreak in wild boar in the district Břeclav closed to the Austrian border after the district Břeclav had been for several years free of CSF. The following two outbreaks in wild boar were recorded in August and September. In December of this year there was an outbreak in big domestic pig farm (582 pigs).

Since December 1995 until now there has been no outbreak of CSF neither in wild boar nor in domestic pigs. The serological investigation of wild boar gives the positive results only in the districts where there were the outbreaks in the past and the incidence of seropositivity decreases from year to year.

The development of the epizootological situation in the Czech Republic is documented in maps and graphics.

- The fact, that CSF occurred after the period of 15 years in October 1990 in the district Břeclav closed to the Austrian border and reappeared in the same area after 5 years (June - July 1995) contributed to common decision of Czech and Austrian veterinary authorities together with European Commission to introduce in this area of Southern Moravia and Lower Austria the common monitoring system supported by E.C. The same common monitoring system will be introduced in the neighbouring areas of Slovakia and Hungary.

## II. Control of CSF

The vaccination of domestic pigs against CSF has been stopped and banned since 1 July 1992. The decision to ban the vaccination of pigs against CSF was not easy because in the year 1992 there were outbreaks of CSF in wild boar in several districts of Southern Moravia which is the most important agricultural area of the Czech Republic. Only in the district Břeclav - one of the 14 districts of the region of Southern Moravia - about 100 000 domestic pigs are kept mostly in farms with hundreds or thousands of pigs.

Since the 1 July only the method stamping out is applied in the case of outbreak of CSF in domestic pigs. The measures against classical swine fever in wild boar were always directed to reduce of wild boar population and at the same time to detection of CSF virus and specific antibodies.

The actual sanitary measures against classical swine fever in wild boar population and in domestic pigs are codified in Decision Nr. 3/1995 of Department of Epizootology of State Veterinary Administration of the Czech Republic of 1 June 1995 and are the following.

#### A. MEASURES IN WILD BOAR POPULATION

1. In the districts with high frequency of seropositive findings in wild boar (more than 10 % of examined wild boars).
  - a) Veterinary examination 100 % of hunted w. b.
  - b) Laboratory examination (virol. and serol.) 50 % of hunted w. b.
2. In the districts with low frequency of seropositive findings in wild boar (lower than 10 % of examined wild boars).
  - a) Veterinary examination 50 % of hunted w. b.
  - b) Laboratory examination (virol. and serol.) 25 % of hunted w. b.
3. The districts neighbouring the districts under the points 1. and 2. and the border districts with Germany, Poland, Slovakia and Austria
  - a) Veterinary examination 20 % of hunted w. b.
  - b) Laboratory examination (virol. and serol.) 10 % of hunted w. b.
4. Other districts
  - a) Veterinary examination 10 % of hunted w. b.
  - b) Laboratory examination (virol. and serol.) 5 % of hunted w. b.

*Throughout the Czech Republic the virological investigation of 100 % of dead found wild boars continue to be performed.*

The fees for hunting wild boar continue at the same rate, e.g. in the districts with serological positive findings 300 Kč per piglet and per last year wild boar, in the districts neighbouring with these districts 300 Kč per piglet only.

The development of wild boar population is presented in graphics.

#### B. MEASURES IN DOMESTIC PIG HERDS

The measures in domestic pig herds are applied in relation with the occurrence of CSF in wild boar population.

1. In the districts with high frequency of seropositive findings in wild boar.

Serological examination in compliance with directive of SVA CR 1992/4 Annex IV (80/217 EEC Annex IV.) in 50 % of pig herds.

Serological examination of all pig movements from breeding and reproductive herds.
2. In the districts with low frequency of seropositive findings in wild boar and in the districts bordering Germany, Poland, Slovakia and Austria.

Serological examination according directive of SVA CR 1992/4 Annex IV (80/217 EEC Annex IV.) in 25 % of pig herds.

Serological examination of all pig movements from breeding and reproductive herds.
3. In the other districts  
Serological examination according directive of SVA CR 1992/4 Annex IV. (80/217 EEC Annex IV.) in 10 % of pig herds.

In the percentage indicated in B/1.2.3 the pig herds with a possible risk (situated closed to the forests, road and other communications) should be included.

In all cases of suspicion of CSF in pigs the virological investigation must be performed according to Annex I/1992/4 (80/217/IV.)

#### C. THE PROTECTION OF PIG HERDS

The decision of the State Veterinary Administration of the Czech Republic Nr. 2/1994 of 1 January 1994 concerning the protection of pig herds against the introducing of CSF is applied.

This decision includes:

for the farms

- 1) Entry of unauthorized persons and vehicles is forbidden.
- 2) Pig farms must be secured (fenced) against penetration of free living (wild boar) or roaming mammals.
- 3) Use of entrance gate disinfection mats is obligatory.
- 4) Keeping in effect the measures prohibiting the entry of vehicles transporting dung from the farm. The dung may be loaded only from outside of the farm.
- 5) Strict hygienic measures for personnel on farms.

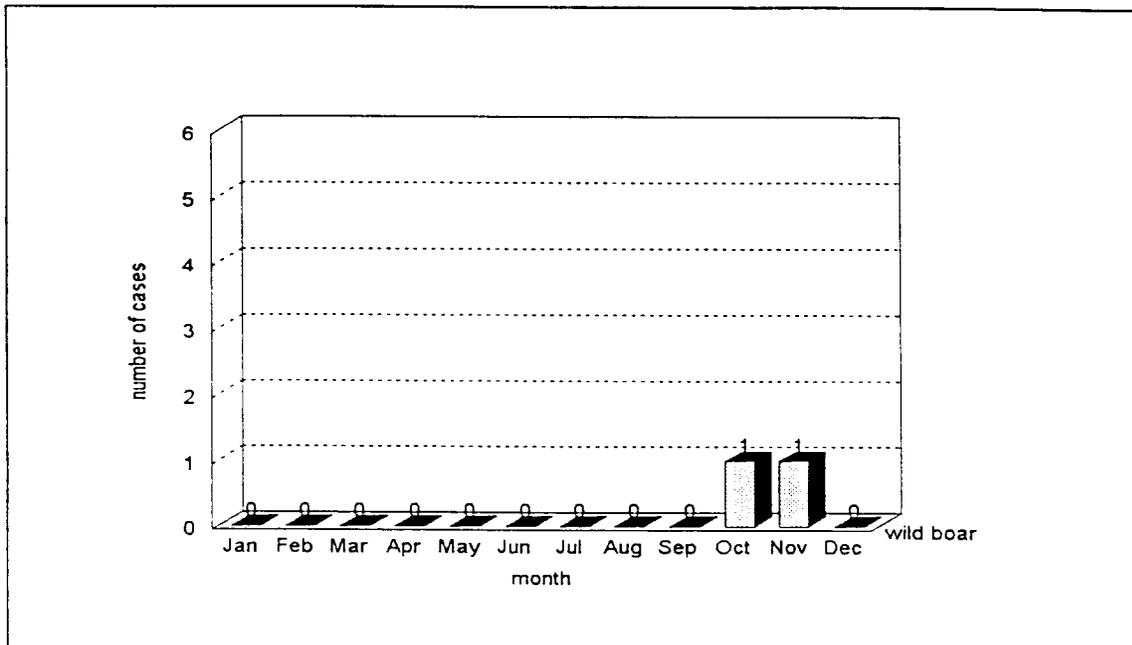
for the pigs

- 6) The movement of pigs from the farms only after veterinary examination and with veterinary certificate is allowed.
- 7) Ban of swill feeding in all pig farms.
- 8) The introducing in the market of ceased meat and organs from slaughterhouse only after the heat treatment is allowed.

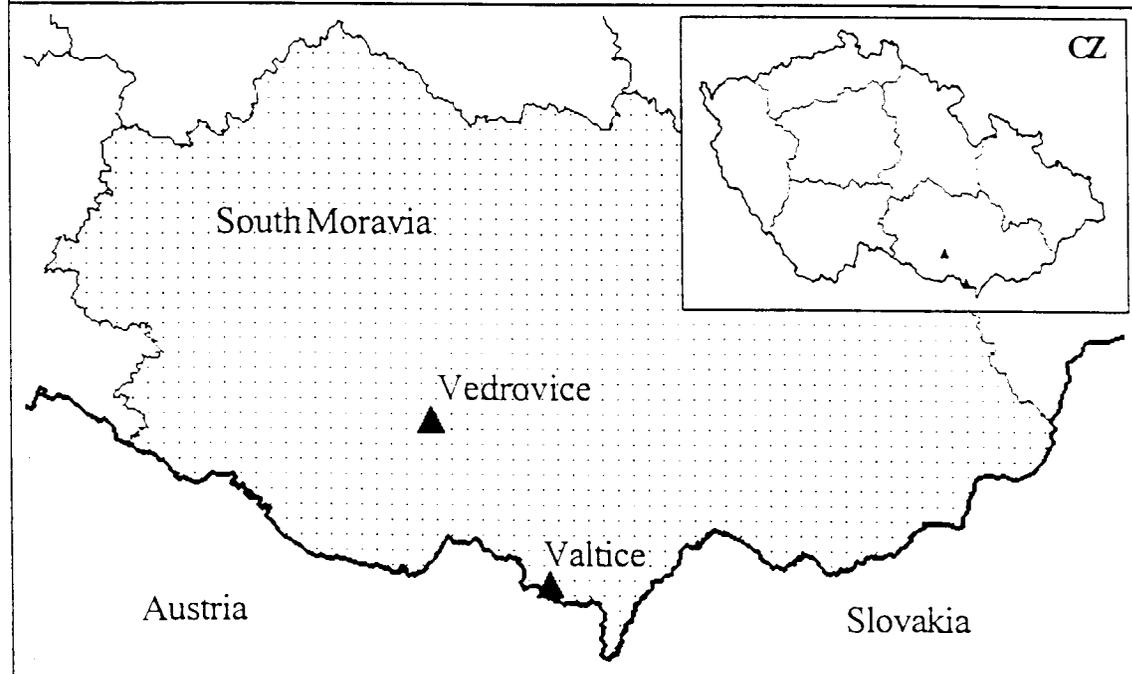
These measures are under permanent control by district veterinary administrations.

**National Swine Fever Laboratory of the Czech Republic  
State Veterinary Institute Jihlava**

Incidence of the classical swine fever during  
January - December 1990

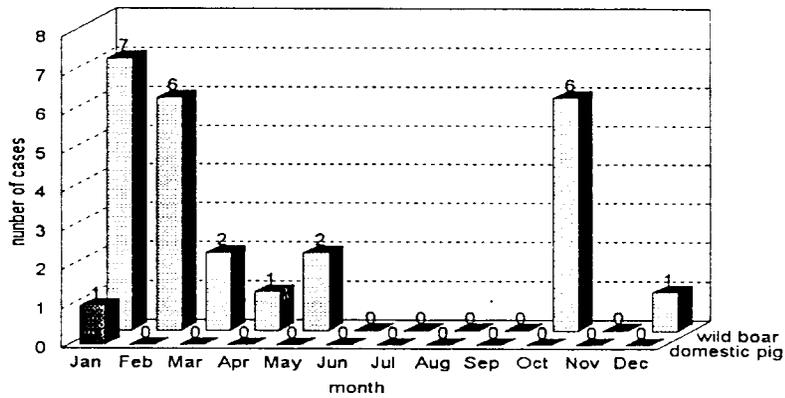


Date	Cadastre name	Animal
30/10/90	Valtice	1 wild boar
01/11/90	Vedrovice	1 wild boar

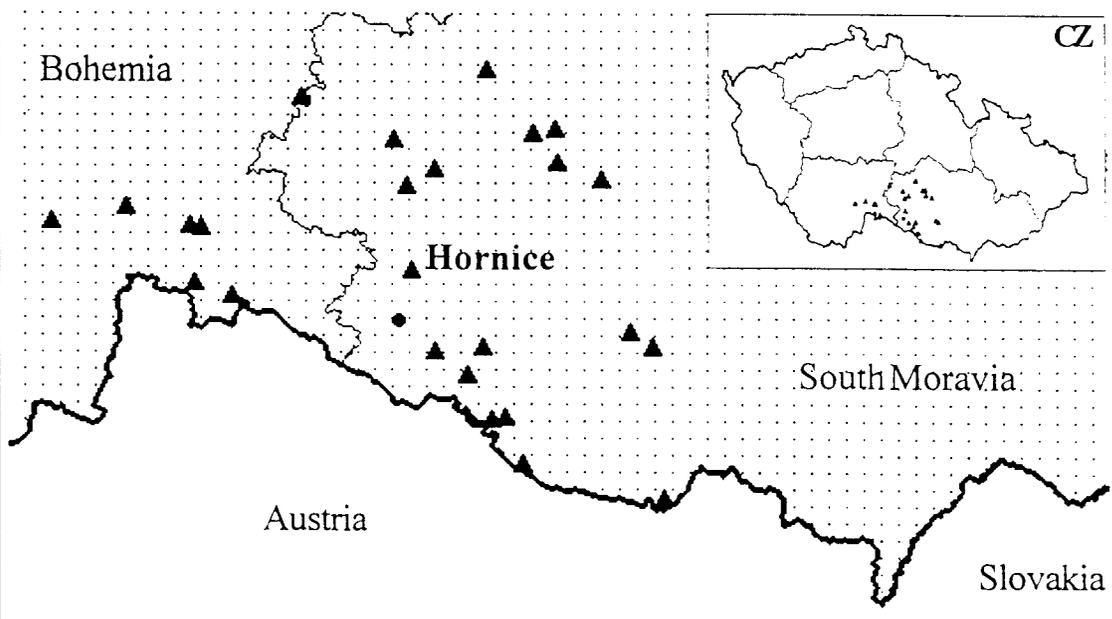


Nation. Swine Fever Laboratory of the Czech Republic  
State Veterinary Institute Jihlava

Incidence of the classical swine fever during  
January - December 1992

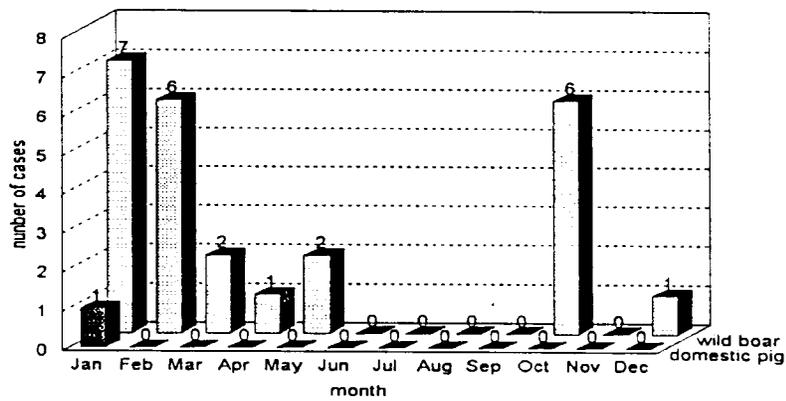


Date	Cadastre name	Animal	Date	Cadastre name	Animal
10/01/92	Šumná	1 wild boar	19/02/92	Hevlín	1 wild boar
10/01/92	Zálesí	1 wild boar	06/03/93	Podmolí	1 wild boar
10/01/92	Pavlice	1 wild boar	06/03/93	Šatov	1 wild boar
13/01/92	<b>Hornice</b>	<b>1 domestic pig</b>	08/04/92	Bukový háj	1 wild boar
14/01/92	Studnice	1 wild boar	06/05/92	Zašovice	1 wild boar
17/01/92	Oslavice	1 wild boar	18/05/92	Lesonice-Domamil	1 wild boar
20/01/92	Arnolec	1 wild boar	02/10/92	Sokoličko	1 wild boar
31/01/92	Baliny	1 wild boar	26/10/92	Klášteř	1 wild boar
03/02/92	Cikov	1 wild boar	26/10/92	Staré Mšsto pod Ladšt.	1 wild boar
13/02/92	Opatov	1 wild boar	27/10/92	Kolenec	1 wild boar
14/02/92	Lesonice	1 wild boar	27/10/92	Čejkov	1 wild boar
14/02/92	Masovice	1 wild boar	29/10/92	Lomy	1 wild boar
14/02/92	Vemyslice	1 wild boar	14/12/92	Komora-Člunek	1 wild boar

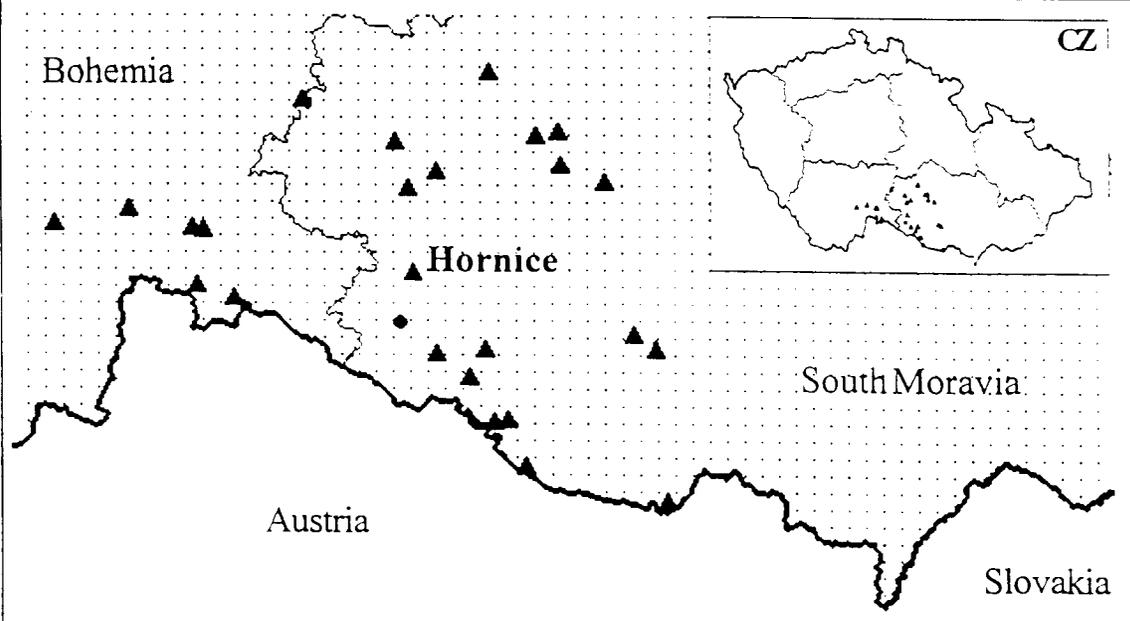


**Nation. Swine Fever Laboratory of the Czech Republic**  
**State Veterinary Institute Jihlava**

Incidence of the classical swine fever during  
 January - December 1992

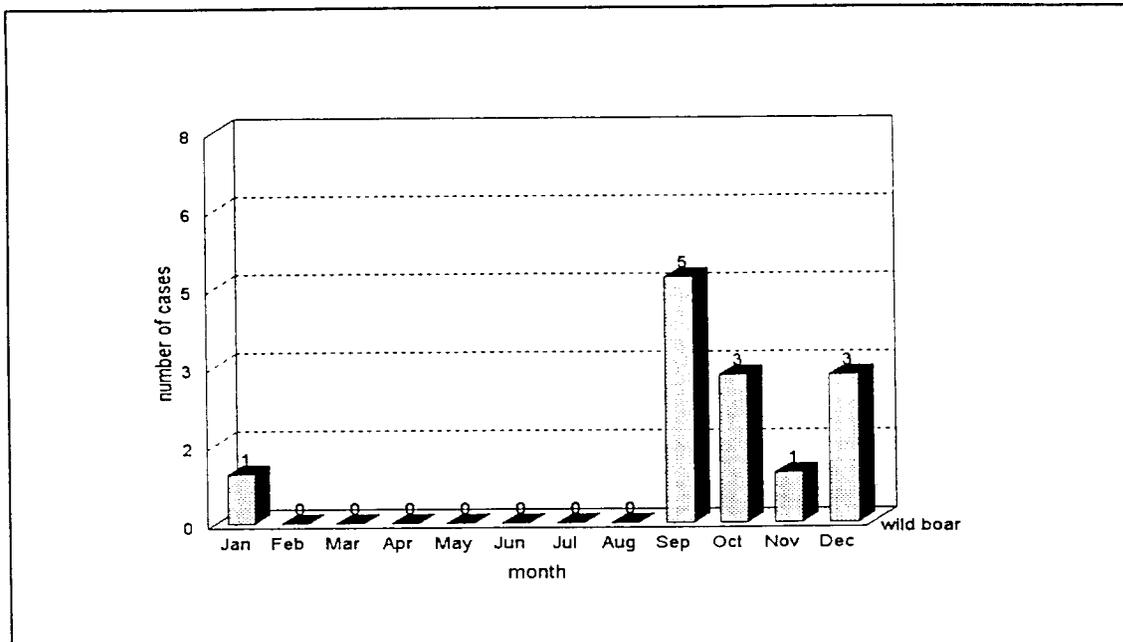


Date	Cadastr name	Animal	Date	Cadastr name	Animal
10/01/92	Šumna	1 wild boar	19/02/92	Hevlin	1 wild boar
10/01/92	Zálesí	1 wild boar	06/03/93	Podmolí	1 wild boar
10/01/92	Pavlice	1 wild boar	06/03/92	Šatov	1 wild boar
13/01/92	<b>Hornice</b>	1 domestic pig	08/04/92	Bukový háj	1 wild boar
14/01/92	Studnice	1 wild boar	06/05/92	Zašovice	1 wild boar
17/01/92	Oslavice	1 wild boar	18/05/92	Lesonice-Domamít	1 wild boar
20/01/92	Arnolec	1 wild boar	02/10/92	Sokoličko	1 wild boar
31/01/92	Baliny	1 wild boar	26/10/92	Klášteř	1 wild boar
03/02/92	Čikov	1 wild boar	26/10/92	Staré Město pod Ladšt.	1 wild boar
13/02/92	Opatov	1 wild boar	27/10/92	Kolenec	1 wild boar
14/02/92	Lesonice	1 wild boar	27/10/92	Čejkov	1 wild boar
14/02/92	Masovice	1 wild boar	29/10/92	Lomy	1 wild boar
14/02/92	Vemyslice	1 wild boar	14/12/92	Komora-Čtunek	1 wild boar

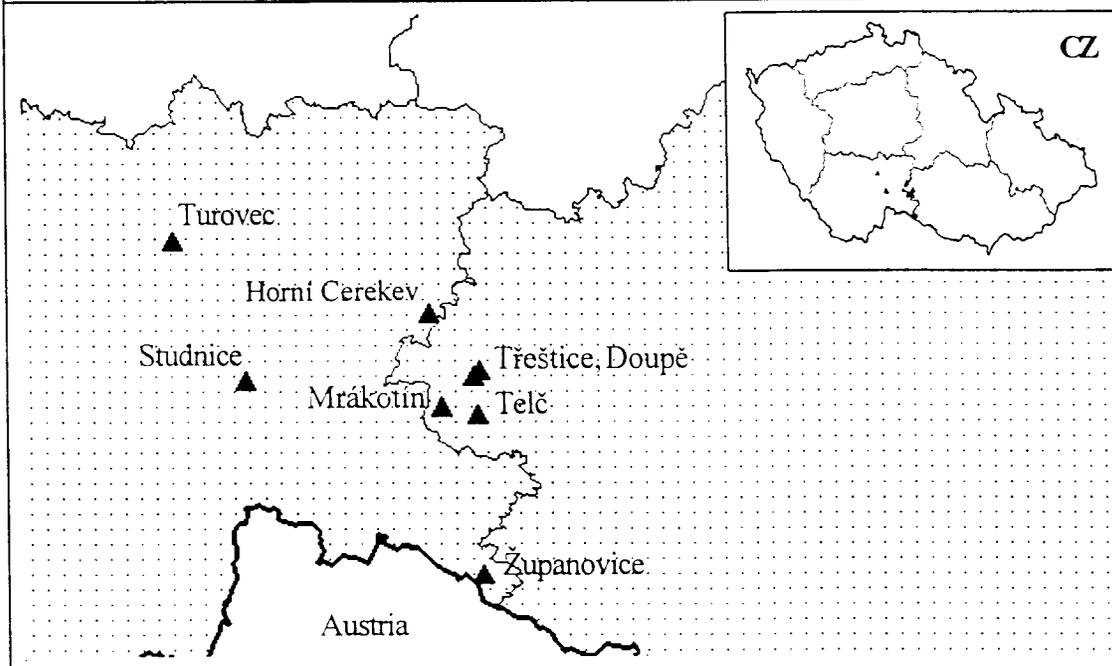


**National Swine Fever Laboratory of the Czech Republic  
State Veterinary Institute Jihlava**

Incidence of the classical swine fever during  
January - December 1993

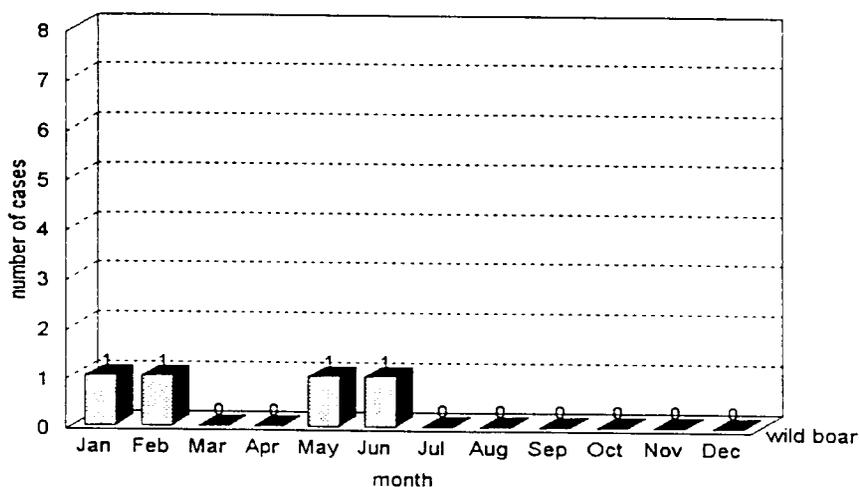


Date	Cadastre name	Animal	Date	Cadastre name	Animal
11/01/93	Županovice u Desné	1 wild boar	09/10/93	Telč - Doupě	1 wild boar
17/09/93	Třeštica	1 wild boar	26/11/93	Telč - Řasná	1 wild boar
22/09/93	Horní Cerekev	3 wild boars	09/12/93	Telč - Lhotka	1 wild boar
24/09/93	Mrákotín	1 wild boar	10/12/93	Turovec	1 wild boar
07/10/93	Telč	2 wild boars	17/12/93	Studnice	1 wild boar



**National Swine Fever Laboratory of the Czech Republic  
State Veterinary Institute Jihlava**

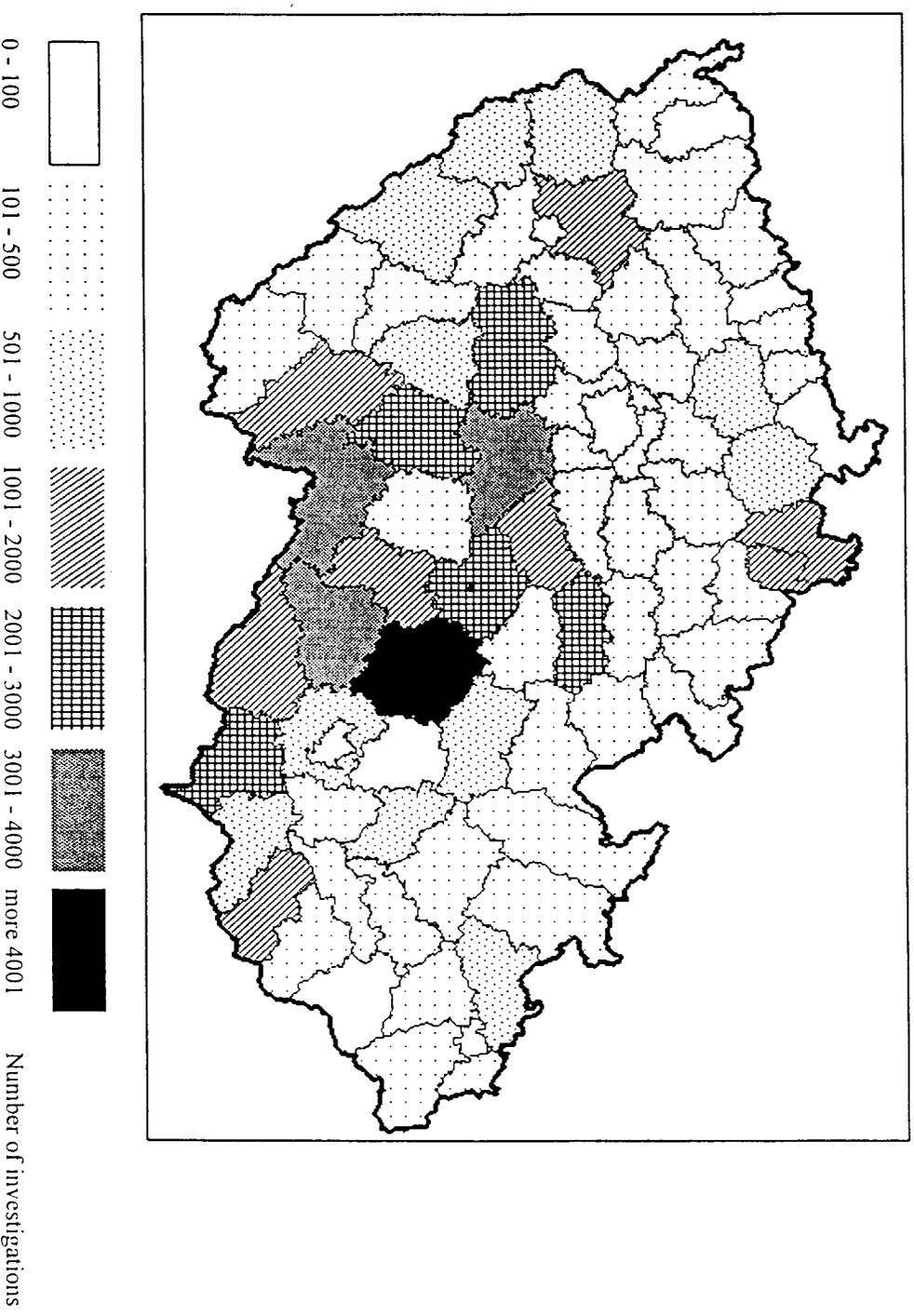
Incidence of the classical swine fever during  
January - December 1994



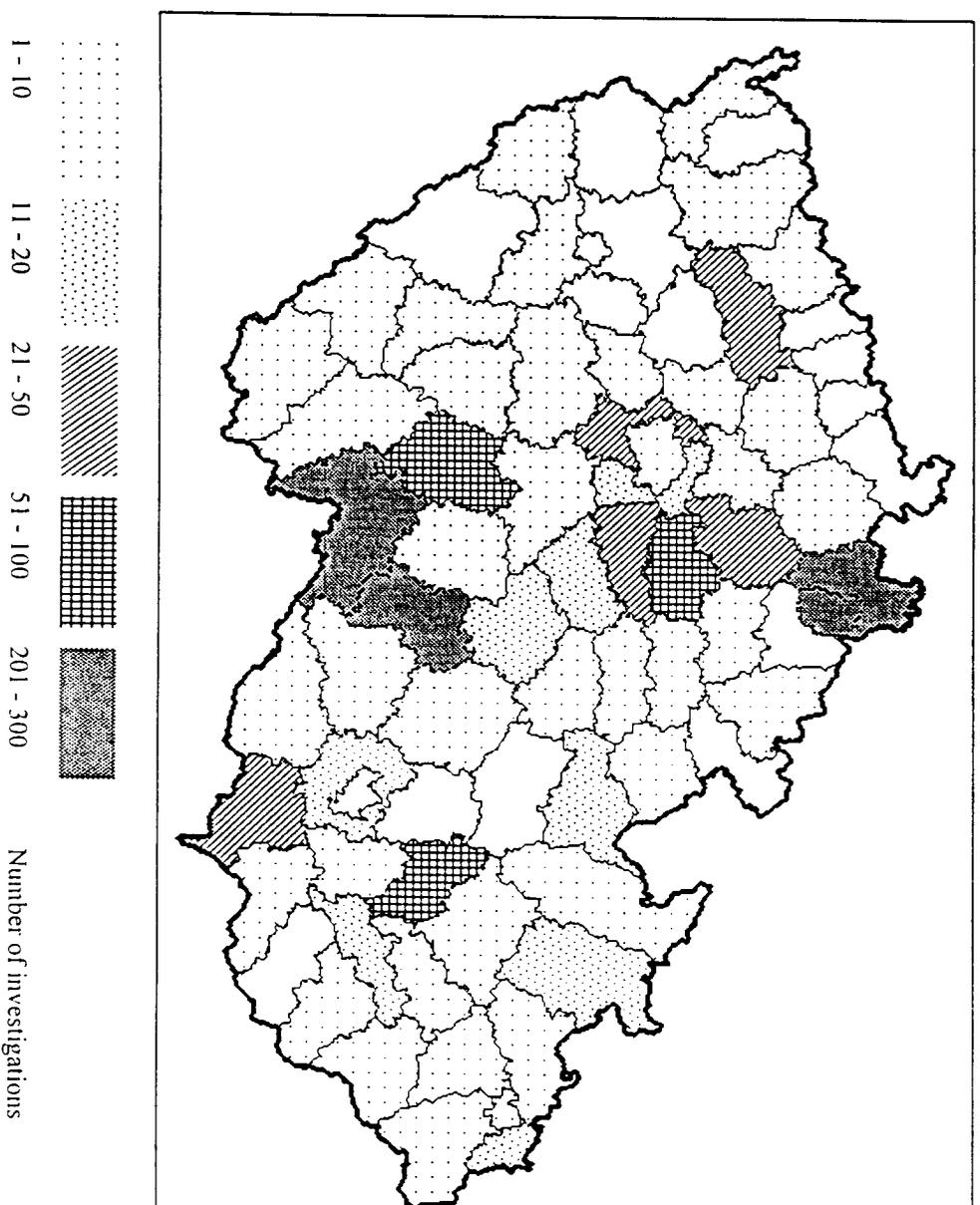
Date	Cadastr name	Animal
12/01/94	Červené Janovice	pathological finding only (virological negativ) in wild boar
24/02/94	Budiškovice	virological positive wild boar
29/06/94	Havlíčkova Borová	virological positive wild boar
03/05/94	Turovec	virological positive domestic pig



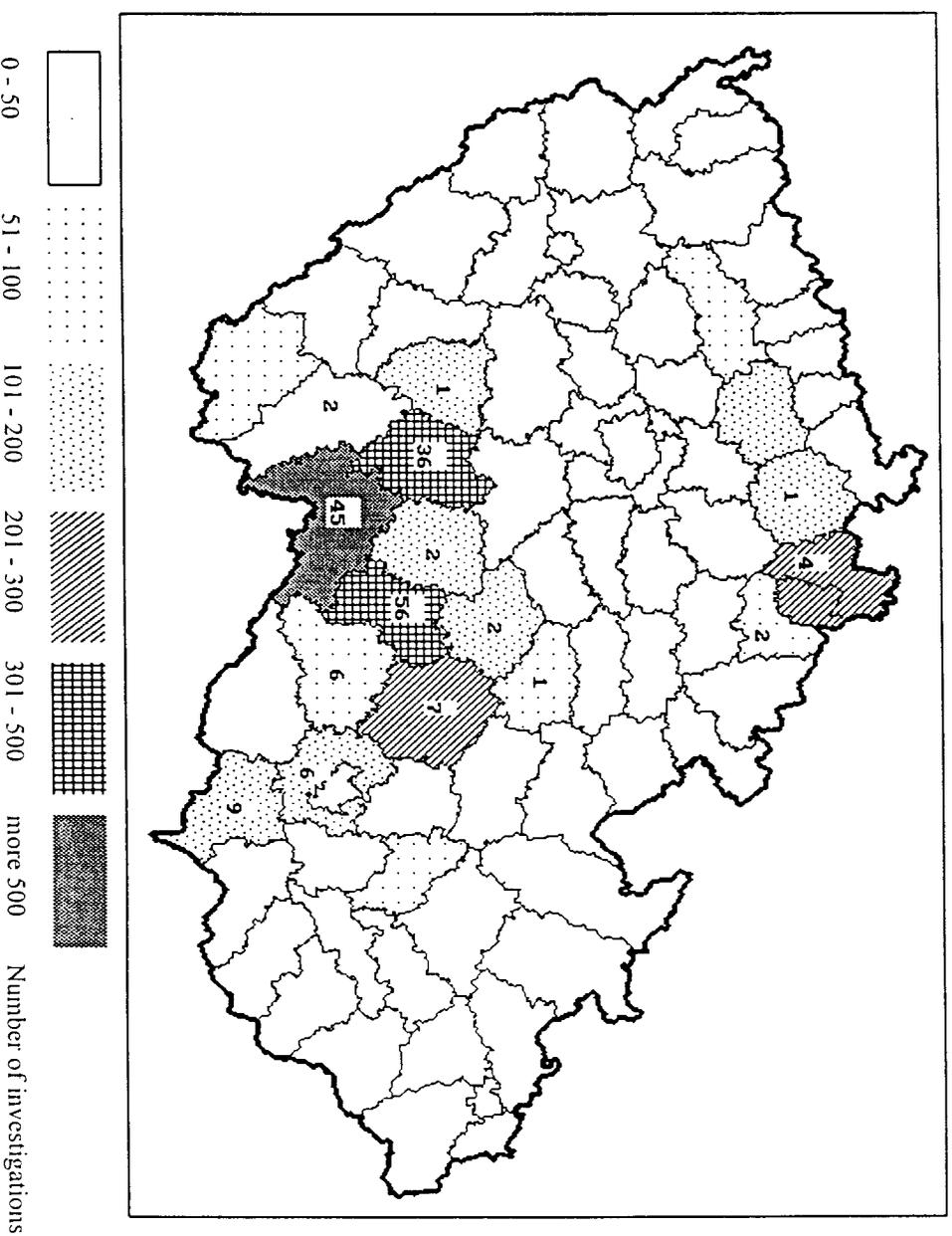
**National Swine Fever Laboratory of the Czech Republic  
State Veterinary Institute Jihlava  
Number of serological investigation of CSF  
in domestic pigs : january - december 1994**



**National Swine Fever Laboratory of the Czech Republic  
State Veterinary Institute Jihlava  
Number of virological investigation of CSF  
in domestic pigs : january - december 1994**



**National Swine Fever Laboratory of the Czech Republic**  
**State Veterinary Institute Jihlava**  
**Number of serological investigations of CSF**  
**in wild boar : january - december 1994**

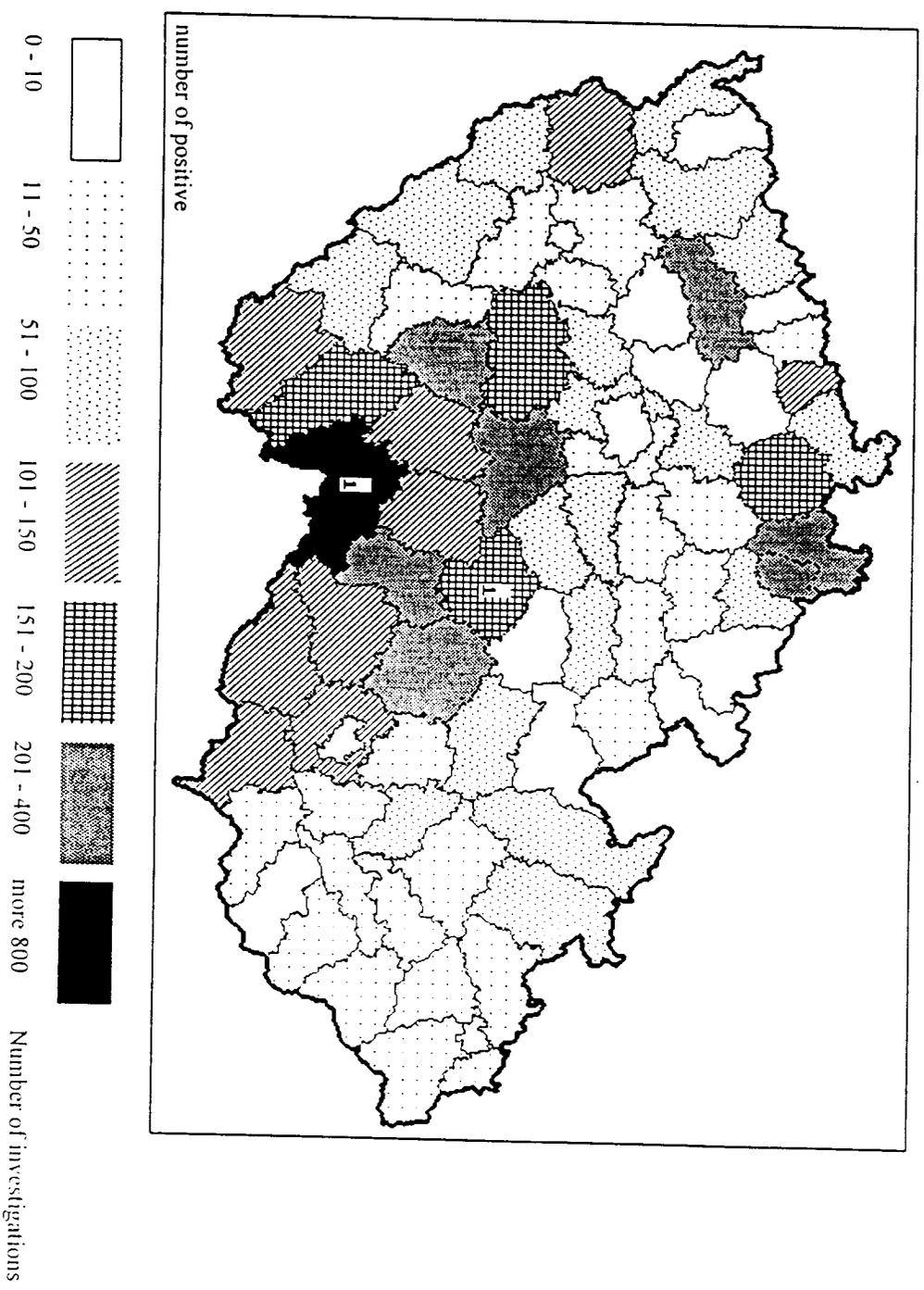


number of serological positive /  
number of serological investigations  
in districts

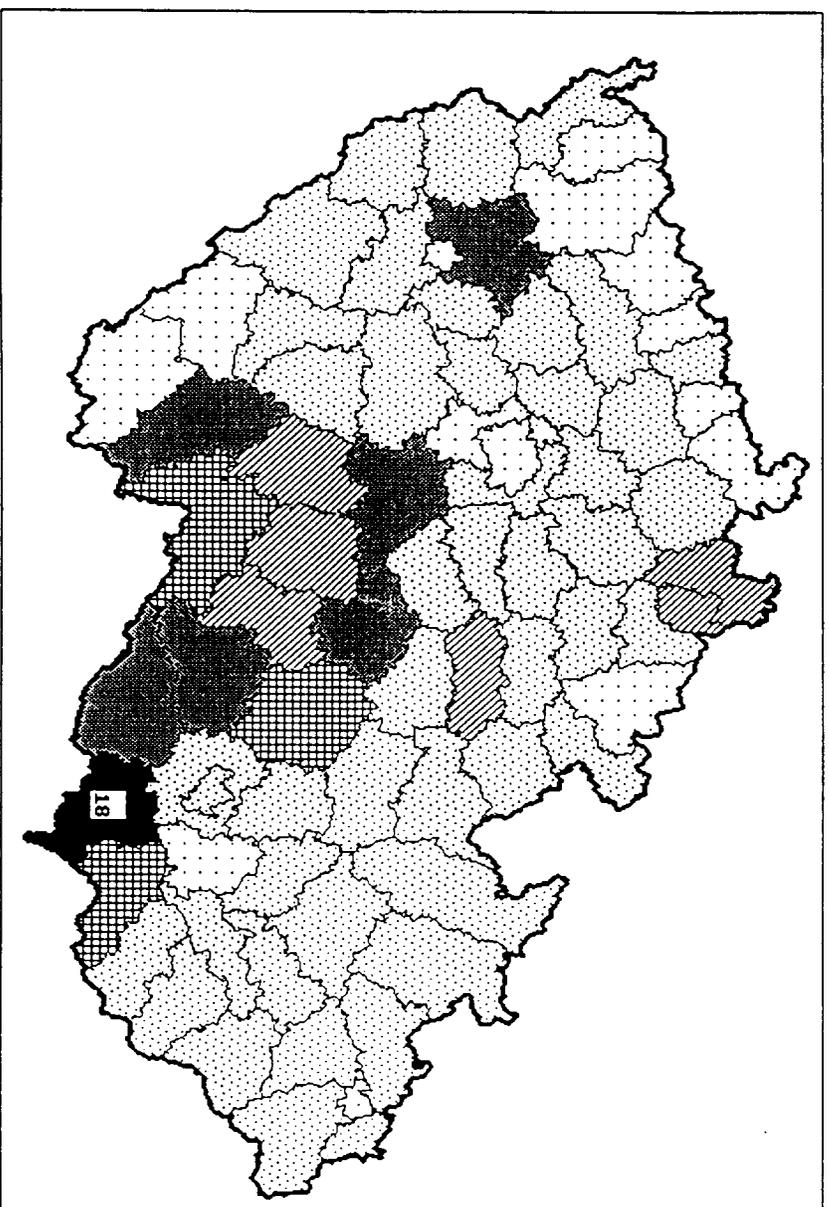
**DISTRICTS**

45 / 501	Jindřichův Hradec
56 / 361	Jihlava
36 / 341	Tábor
7 / 234	Žďár nad Sázavou
4 / 204	Liberec
6 / 146	Brno venkov
9 / 132	Břeclav
1 / 123	Písek
2 / 117	Pelhřimov
2 / 108	Havlíčkův Brod
1 / 101	Česká Lípa
2 / 74	Semily
1 / 71	Chrudim
6 / 68	Třebíč
2 / 24	České Budějovice

National Swine Fever Laboratory of the Czech Republic  
State Veterinary Institute Jihlava  
Number of virological investigation of CSF  
in wild boar : january - december 1994



National Swine Fever Laboratory of the Czech Republic  
 State Veterinary Institute Jihlava  
 Number of serological investigations of CSF  
 in domestic pigs : january - december 1995



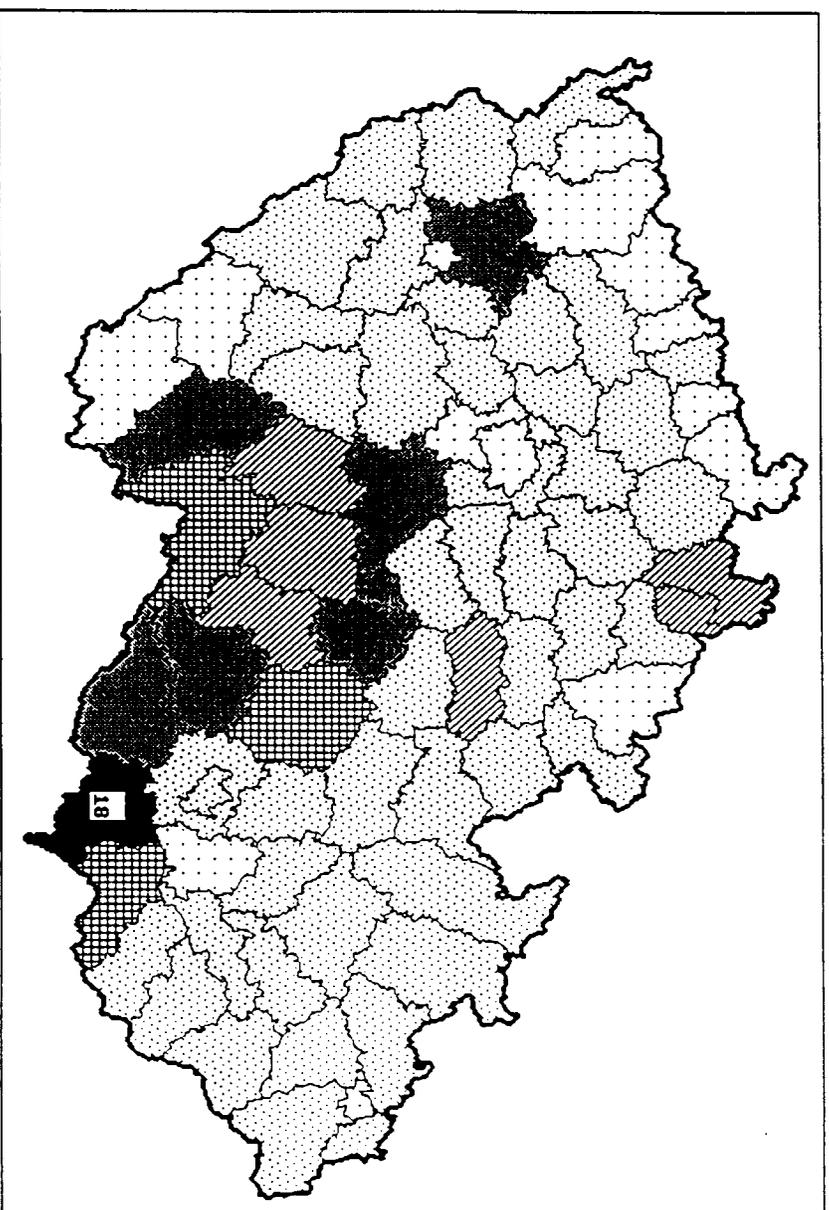
number of serological positive /  
 number of serological investigations  
 in districts

DISTRICT

18 / 6537 Břeclav

0 - 100    101 - 1000    1001 - 2000    2001 - 3000    3001 - 4000    more 6000    Number of investigations

National Swine Fever Laboratory of the Czech Republic  
 State Veterinary Institute Jihlava  
 Number of serological investigations of CSF  
 in domestic pigs : january - december 1995



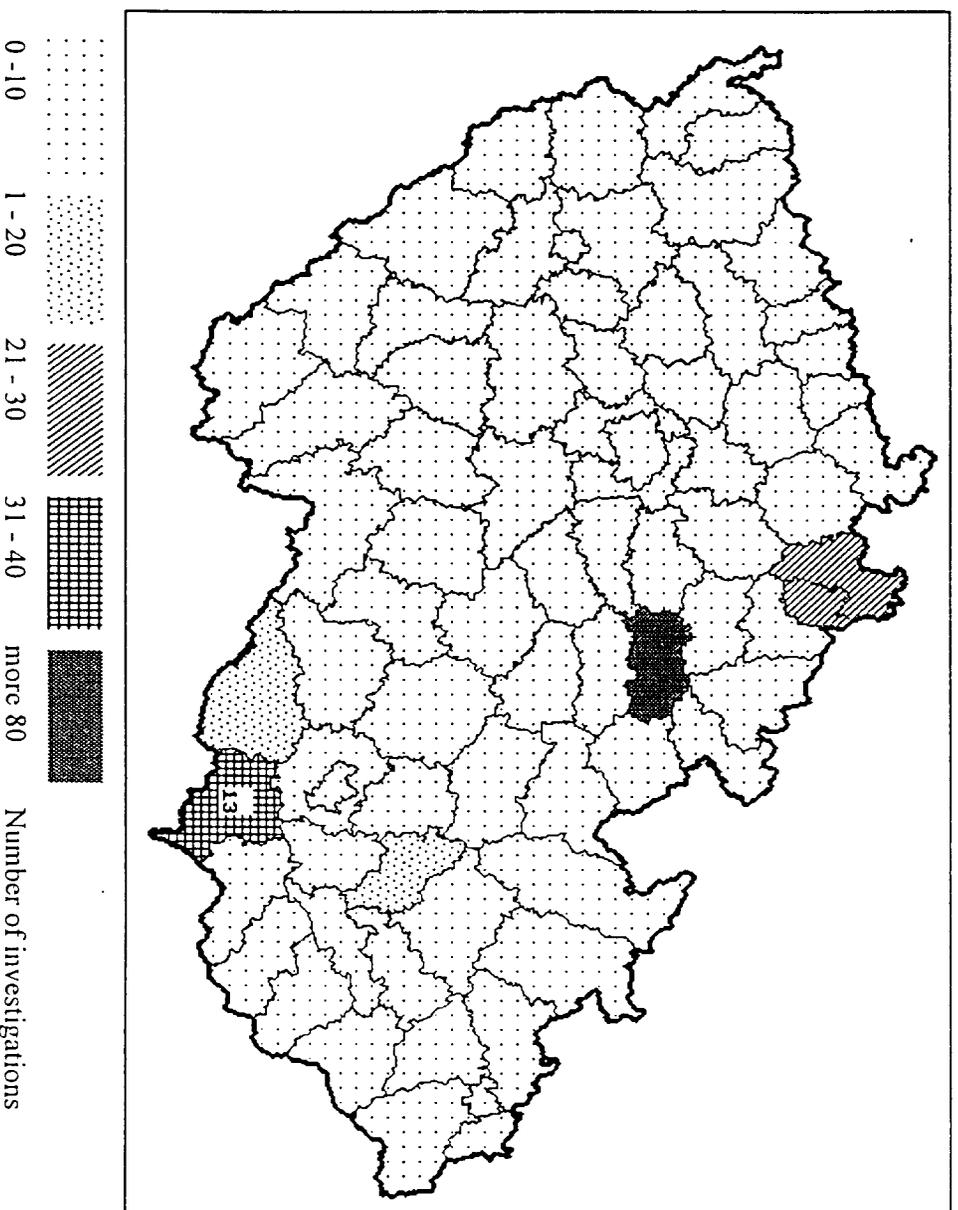
0 - 100    101 - 1000    1001 - 2000    2001 - 3000    3001 - 4000    more 6000    Number of investigations

number of serological positive /  
 number of serological investigations  
 in districts

DISTRICT

18 / 6537    Břeclav

**National Swine Fever Laboratory of the Czech Republic**  
**State Veterinary Institute Jihlava**  
**Number of virological investigations of CSF**  
**in domestic pigs : january - december 1995**

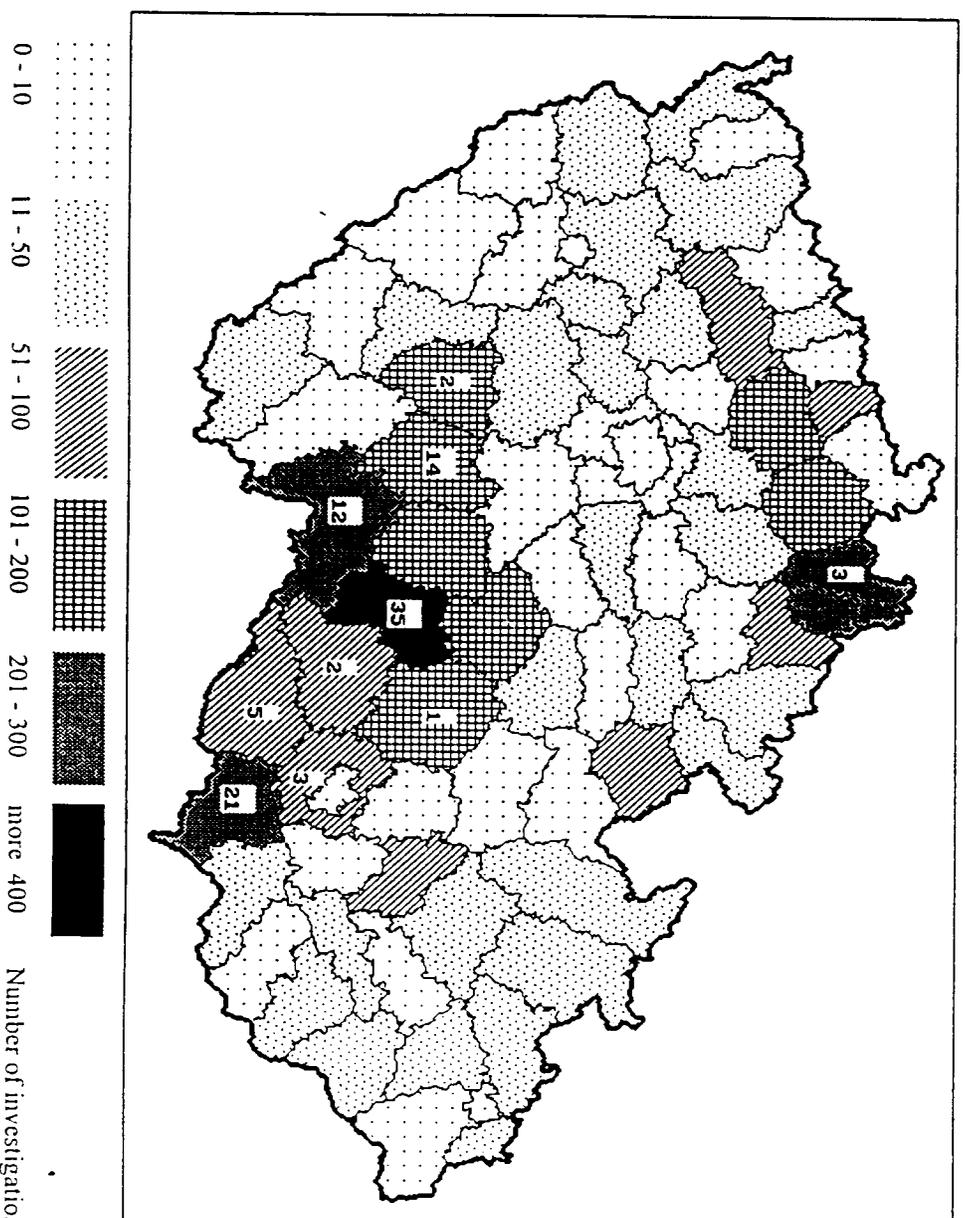


number of virological positive /  
 number of virological investigations  
 in districts

DISTRICT

13 / 34      Břeclav

**National Swine Fever Laboratory of the Czech Republic**  
**State Veterinary Institute Jihlava**  
**Number of serological investigations of CSF**  
**in wild boar : January - december 1995**

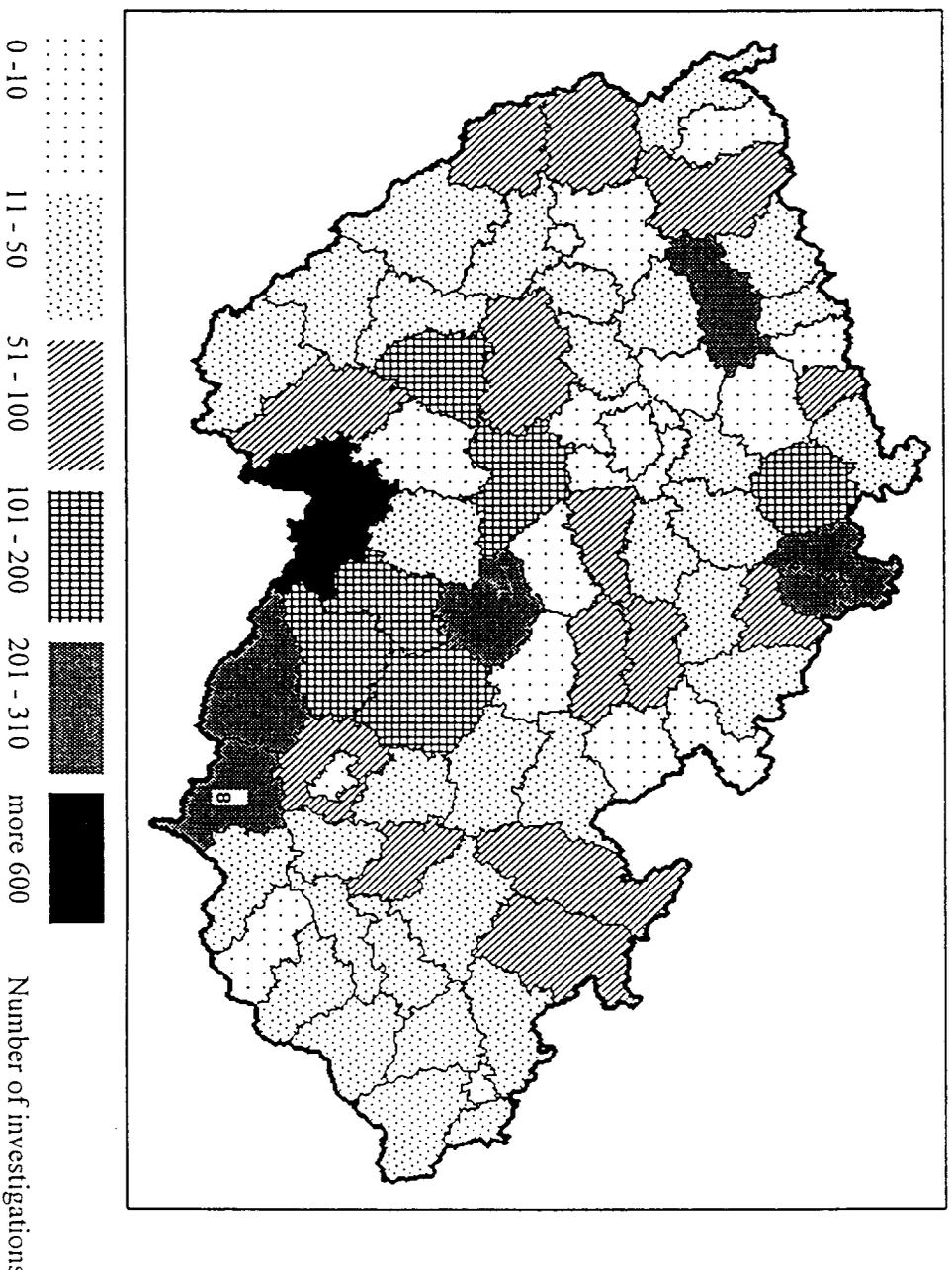


number of serological positive /  
number of serological investigations  
in districts

**DISTRICTS**

3 / 99	Brno - venkov
21 / 244	Břeclav
35 / 403	Jihlava
12 / 266	Jindřichův Hradec
3 / 295	Liberec
14 / 137	Tábor
2 / 65	Třebíč
5 / 99	Znojmo
1 / 194	Žďár nad Sázavou

National Swine Fever Laboratory of the Czech Republic  
 State Veterinary Institute Jihlava  
 Number of virological investigations of CSF  
 in wild boar : January - december 1995



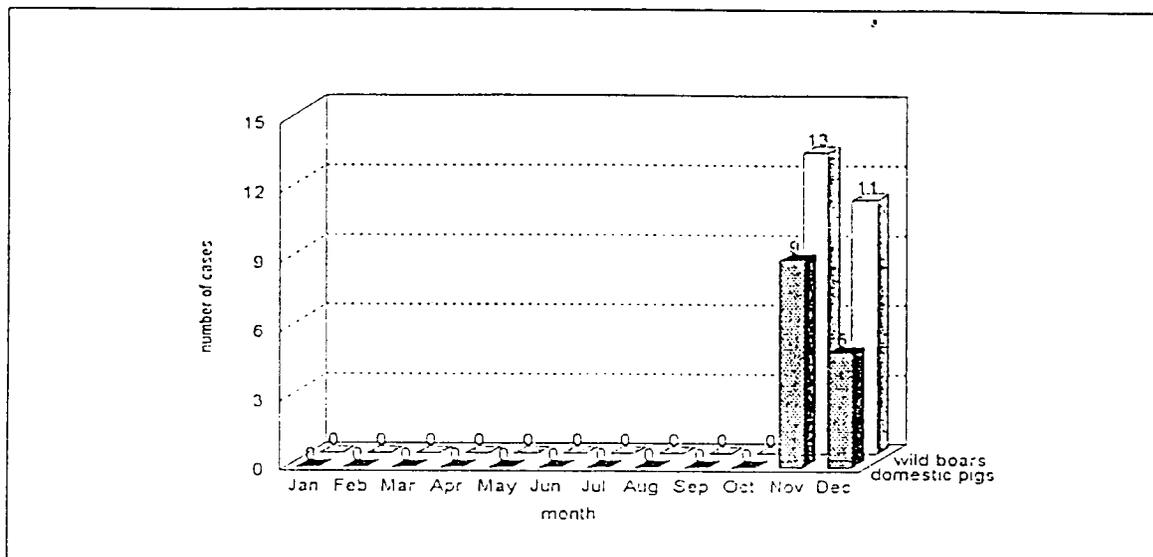
number of virological positive /  
 number of virological investigations  
 in districts

DISTRICT

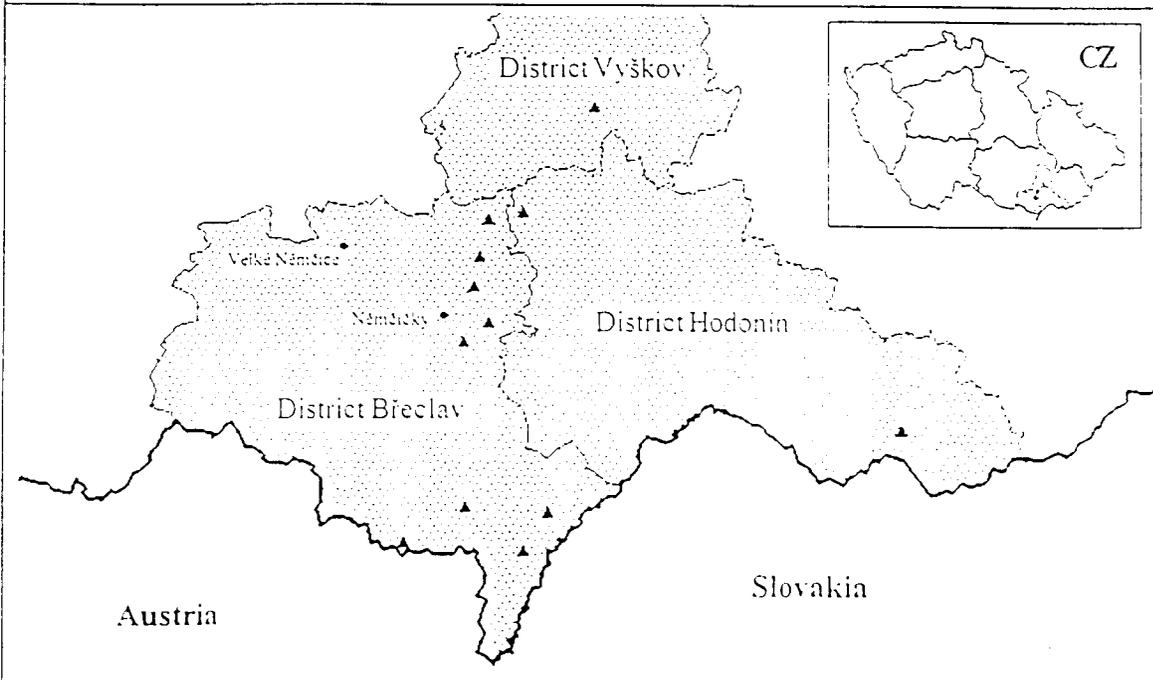
8 / 289      Břeclav

National Swine Fever Laboratory of the Czech Republic  
State Veterinary Institute Jihlava

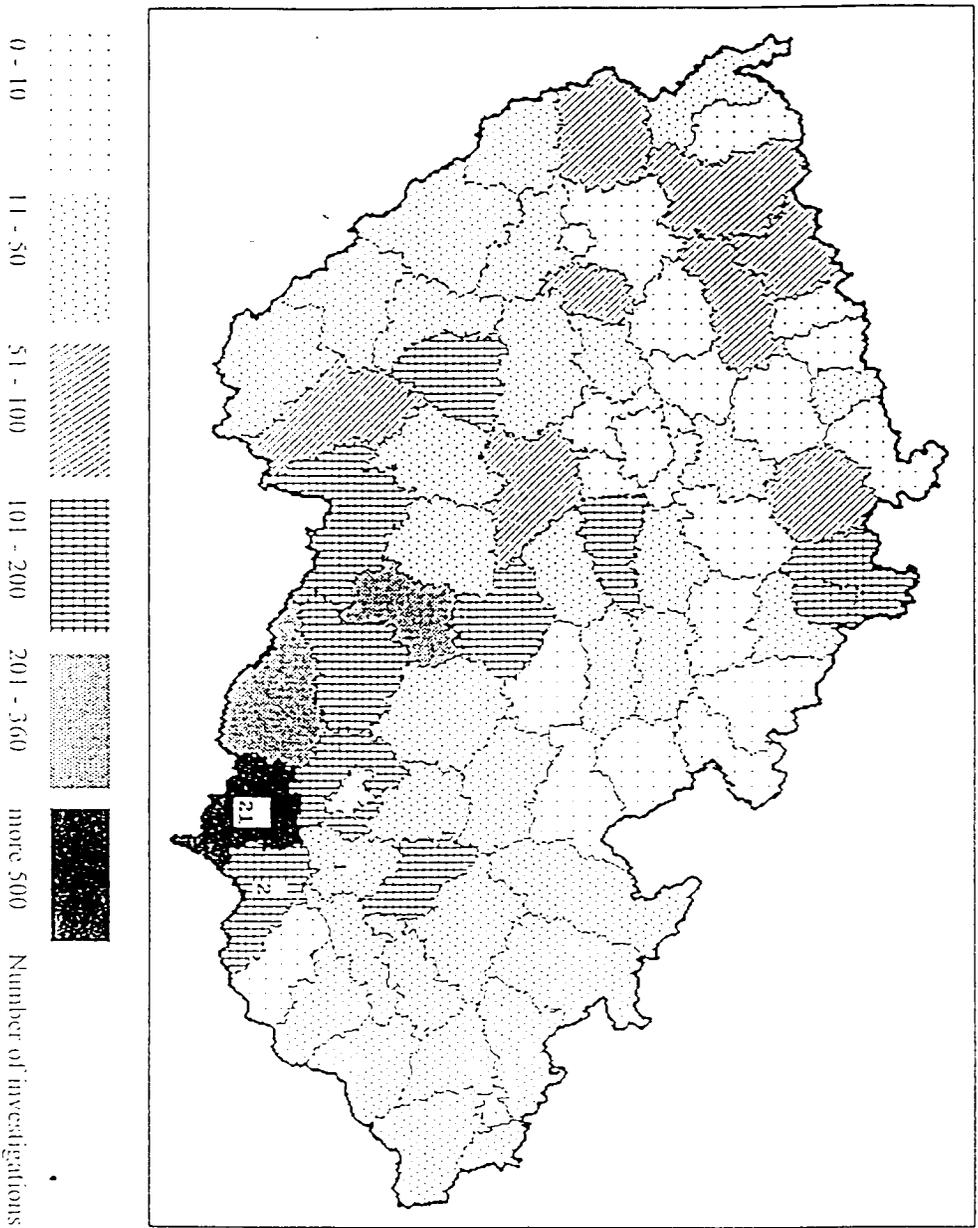
Incidence of the classical swine fever during  
January - December 1996



Date	Cadastre name	Animal	Date	Cadastre name	Animal
11/96	Pohansko - Lanžhot	2 wild boars	12/96	Morkůvsky	1 wild boars
11/96	Pohansko - Břeclav	5 wild boars	12/96	Tvrdonice	1 wild boars
11/96	Valtice	1 wild boar	12/96	Pohansko - Břeclav	1 wild boar
11/96	Kobylna na Moravě	1 wild boar	12/96	Bořetice u Hustopečí	3 wild boar
11/96	Morkůvsky	1 wild boar	12/96	Klobouky u Brna	1 wild boar
11/96	Klobouky u Brna	1 wild boar	12/96	Velké Němčice	5 domestic pigs
11/96	Velké Hostěradky	1 wild boar	12/96	Dambořice	1 wild boar
11/96	Lanžhot	1 wild boar	12/96	Hrubá Vrbka	1 wild boar
11/96	Němčický	9 domestic pigs	12/96	Bušovice	1 wild boar
			12/96	Lanžhot	1 wild boar



National Swine Fever Laboratory of the Czech Republic  
 State Veterinary Institute Jihlava  
 Number of virological investigation of CSF  
 in wild boar : January - december 1996

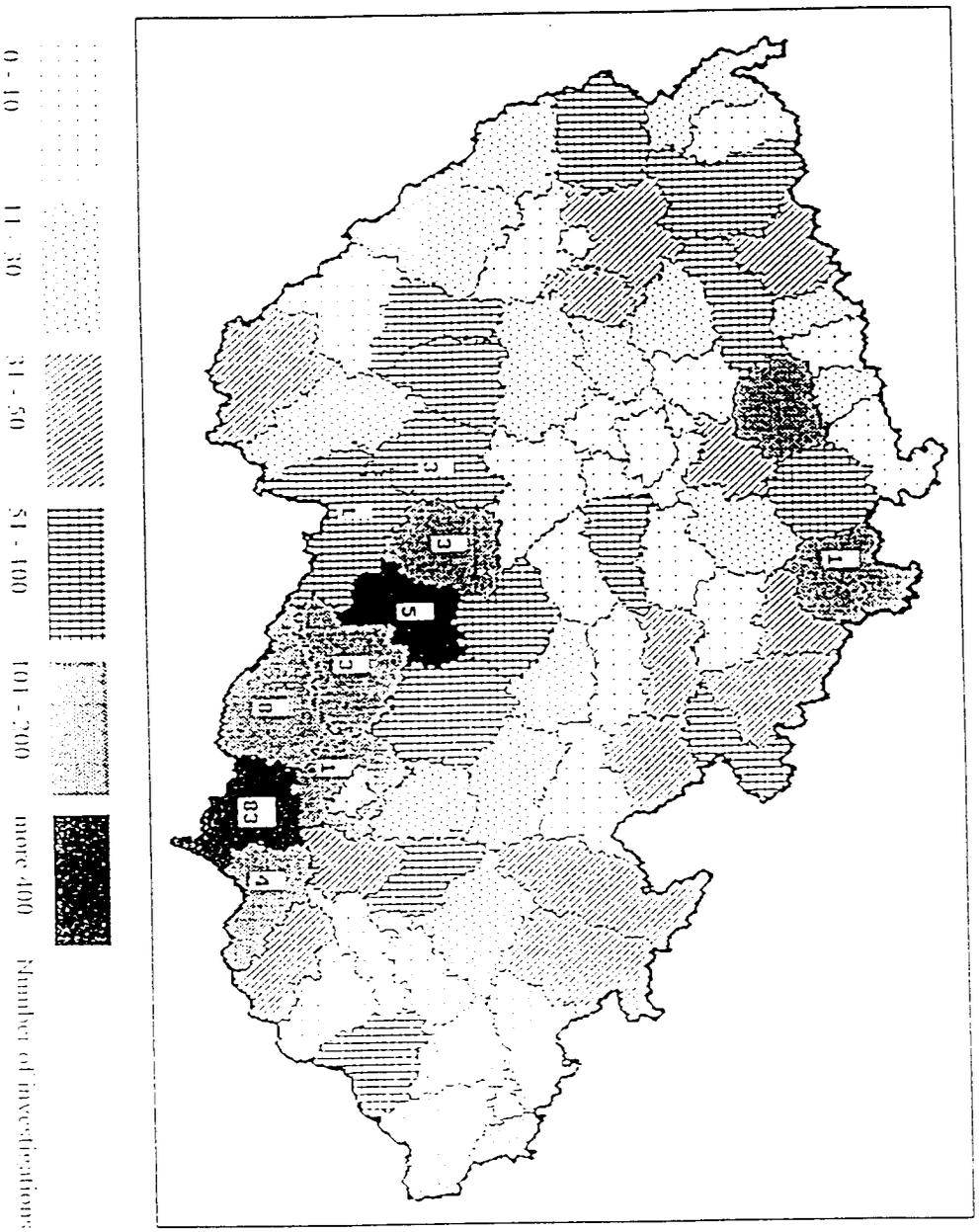


number of virological positive /  
 number of virological investigations  
 in districts

DISTRICTS

21 / 527	Břeclav
2 / 157	Hodonín
1 / 47	Vyškov

National Swine Fever Laboratory of the Czech Republic  
 State Veterinary Institute Jihlava  
 Number of serological investigation of CSF  
 in wild boar : january - december 1996



number of serological positive /  
 number of serological investigations  
 in districts

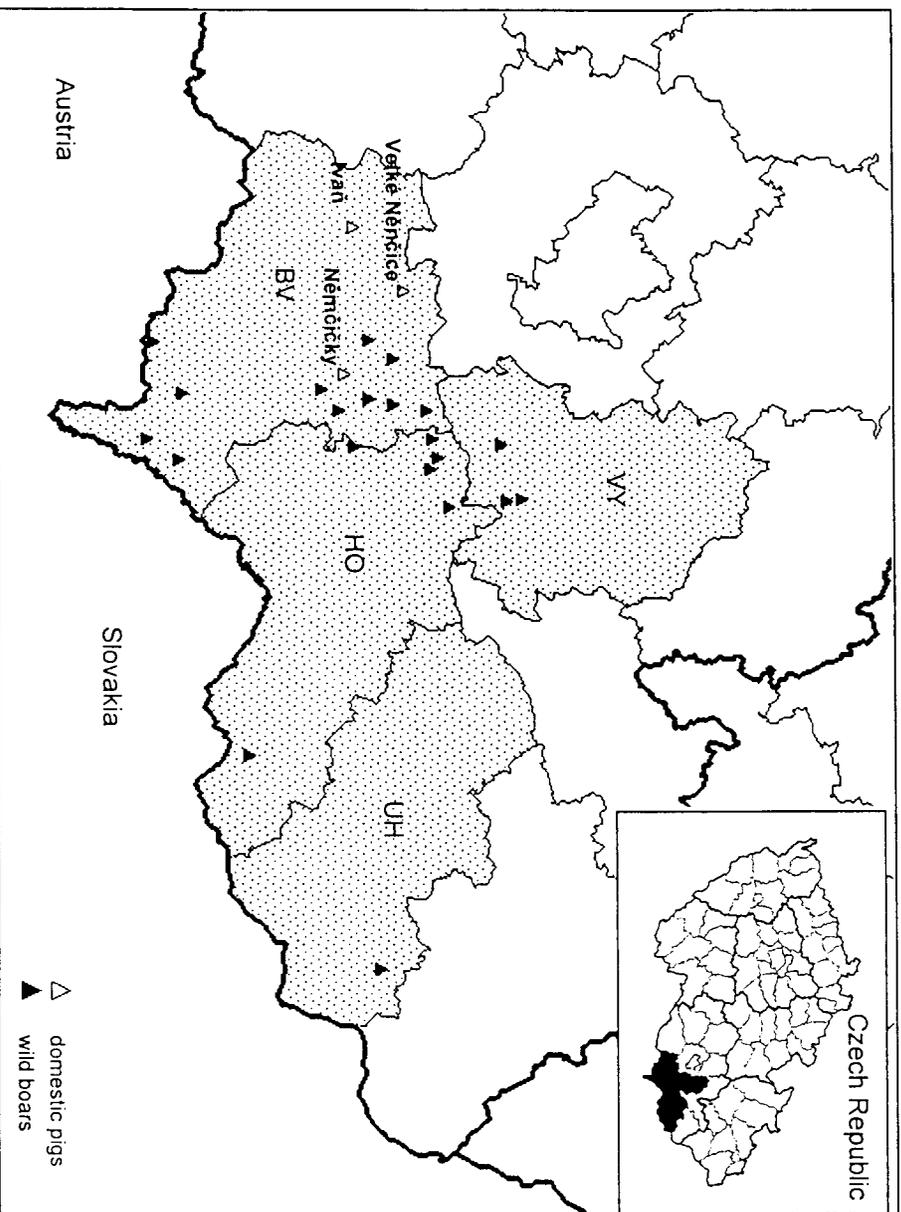
**DISTRICTS**

1 / 125	Brno - venkov
83 / 467	Břeclav
4 / 110	Hodonín
5 / 430	Jihlava
1 / 85	Jindřichův Hradec
1 / 143	Labetov
3 / 115	Pelhřimov
3 / 83	Tábor
3 / 134	Třebíč
8 / 151	Znojmo

# CLASSICAL SWINE FEVER - LABORATORY INVESTIGATION RESULTS

november 1996 - february 1997

## Positive virological investigations

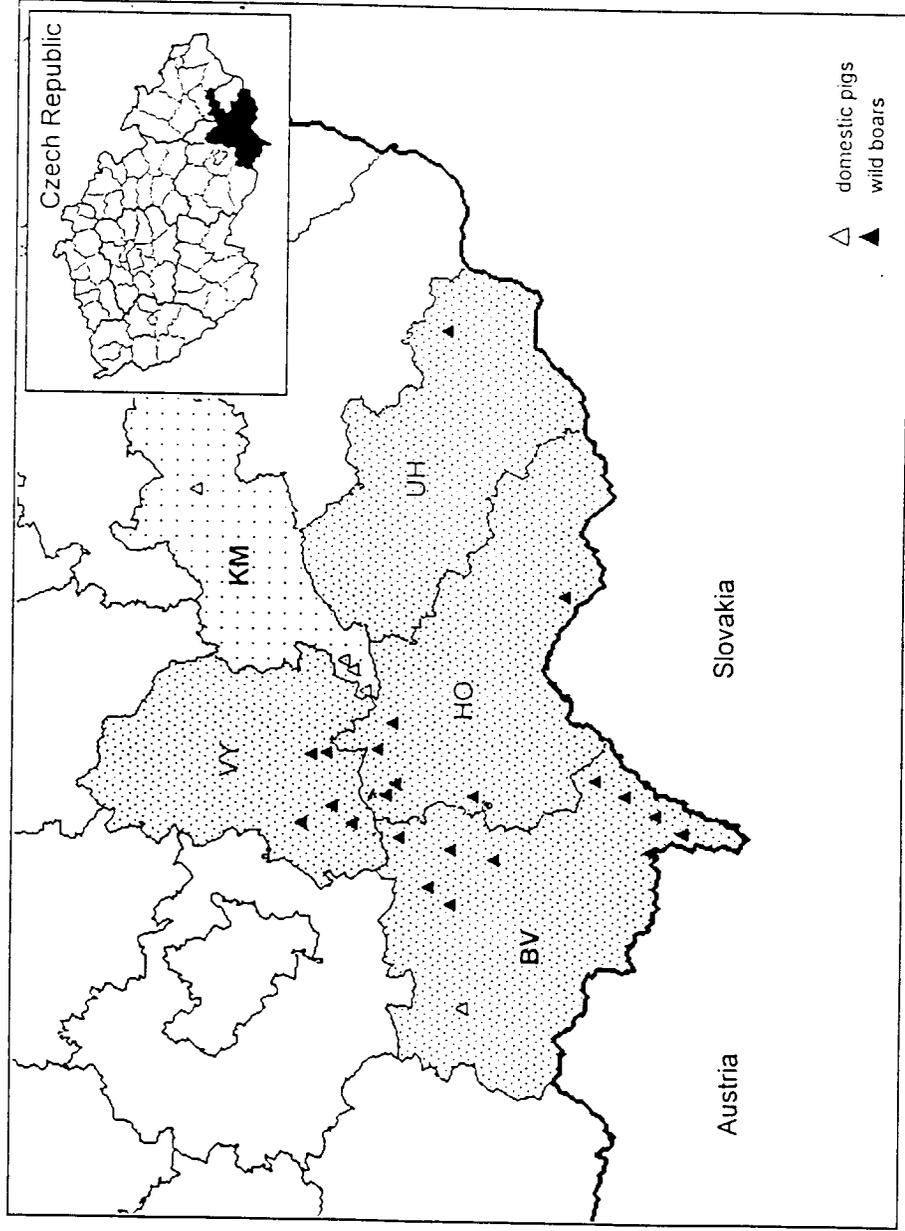


Date	Cadastre Name	Animal	Posit. virol. inv.
11/96	Pohansko-Lanžhot	wild boars	2
11/96	Pohansko- Břeclav	wild boars	5
11/96	Valtice	wild boar	1
11/96	Kobylí na Moravě	wild boar	1
11/96	Morkůvky	wild boar	1
11/96	Klobouky u Brna	wild boar	1
11/96	Velké Hostěrádky	wild boar	1
11/96	Lanžhot	wild boar	1
11/96	Němčický	domestic pigs	9
12/96	Morkůvky	wild boar	1
12/96	Tvrdonice	wild boar	1
12/96	Pohansko- Břeclav	wild boar	1
12/96	Bořetice u Hustop.	wild boars	3
12/96	Klobouky u Brna	wild boar	1
12/96	Velké Němčice	domestic pigs	5
12/96	Dambořice	wild boar	1
12/96	Hrubá Vrbka	wild boar	1
12/96	Bučovice	wild boar	1
12/96	Lanžhot	wild boar	1
1/97	Kurdějov	wild boars	4
1/97	Diváky	wild boar	1
1/97	Uhřetice	wild boars	7
1/97	Klobouky	wild boar	1
1/97	Zaroušice	wild boar	1
1/97	Herspice	wild boars	2
1/97	Zdánice	wild boar	1
2/97	Bojkovice	wild boar	1
2/97	Terezín u Čelče	wild boar	1
2/97	Ivaň	domestic pigs	2

# CLASSICAL SWINE FEVER - LABORATORY INVESTIGATION RESULTS

January - June 1997

Positive virological investigations



District	Animal	Posit. virol. inv.
BV Břeclav	wild boars	23
HO Hodonin	wild boars	11
UH Uh. Hradiště	wild boar	1
VY Vyškov	wild boars	20
BV Břeclav	wild boars	55
Ivaň	domestic pigs	2
KM Kroměříž	domestic pigs	8
Jestřabice	21.2.97	
Liskovec	7.6.97	
Koryčany	7.6.97	
Hulín	12.6.97	
<b>domestic pigs</b>		<b>10</b>
<b>Total</b>		<b>65</b>