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AN INVESTIGATION AND CHARACTERIZATION OF ENTEROVIRUS STRAINS IN SWEDISH PIGS

II. PATHOGENICITY TESTS AND SEROLOGICAL PROPERTIES[1]).

By

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The isolation of the frequently occurring enterovirus strain S180/4, which is antigenically different from the Teschen viruses and of strain S159 from Swedish pigs, has been reported previously (12, 13, 17, 18). In part I of this paper the isolation of 16 ether-resistant viruses in altogether 60 faecal specimens, each representing one herd, was described (20). Other authors' results also show that there are several swine enteroviruses which are different (7, 10, 13, 23).

The isolation and purification, as well as morphological and biological properties in swine kidney tissue culture (t. c.), particle size, sensitivity to storage, and resistance to ether and chloroform, were reported in part I (20).

One of the isolates, U6, which proved to belong to the Teschen group, was described earlier in connection with a disease outbreak in Swedish swine herd (19).

The serological relationship between the 16 isolates and strains S180/4, S159, and the Talfan strain was preliminarily examined by neutralization tests. Those isolates which were obviously different from the above-mentioned strains were selected for further examination, namely for the pathogenic properties in colostrum-deprived piglets and in mice, the duration of the eli-

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mination via the faeses in piglets, and the cytopathogenic effect (CPE) on t. c. from different animals. The antibody content in serum of pigs from which the viruses were isolated, was determined against the own strain. The serological relationship between the strains was examined. The results will be described here.

MATERIAL AND METHODS

Preparation of the t.c. has been described earlier (20). Kidneys were taken from 4—5 week old piglets, dogs and calves, 2 week old rabbits and guinea pigs, and 1 week old albino mice and rats. The so-called chick embryo fibroblast cultures were prepared from chick embryos which had been incubated for 14 days. Skin, extremities, head, and internal organs were removed and the remainder was trypsinized. Human kidney and liver t. c. were kindly provided by the Virus Department of the Karolinska Institute in Stockholm.

The growth medium contained Hanks' BSS, hydrolyzed lactalbumin 0.5 %, and calf serum 5—7.5 %. The serum was omitted in the maintenance medium, except for guinea-pig, rabbit, mouse and rat t. c., where 2 % calf serum was used.

The viruses used were 6 strains, designated as N7, N11, U1, U6, U10, and V4. They had been isolated from faeces of normal pigs, and had been selected as described above.

Before use, the viruses went through 6 t. c. passages, including 3 with the limit dilution procedure. Before some experiments, *e. g.* the neutralization tests, they were carried through 12 passages, that is, in addition to the prementioned ones, through another 6 passages, in 3 of which the plaque purification procedure was also for the 26th and 22nd t. c. passages of strains S180/4 and S159. This done, these strains had thus gone through 32 and 28 t. c. passages, respectively.

The Talfan strain was kindly provided by Dr. A. Mayr, Federal Research Institute for Animal Virus Diseases, Tübingen, Germany.

The preparation and storage of virus suspension has been described earlier (20).

Pathogenicity tests

Mice. Strains N7, N11, U1, U6, U10, V4, S180/4, and S159, purified by the limit dilution procedure, were inoculated on suckling

mice, not older than 24 hours. One litter, that is 3—6 animals, was used for each strain. 0.01 ml. of virus suspension was given intracerebrally, and at the same time, 0.2 ml. intraperitoneally. After 4—5 days one mouse was killed by ether inhalation, and 20 % tissue suspensions was prepared from the brain and from the pooled organs of the pleural and peritoneal cavities, respectively. The suspensions were inoculated on swine kidney t. c., 0.2 ml. per tube. The same was done with a few mice which died within 24 hours after the inocualtion. The surviving inoculated mice were observed for at least 2 weeks.

One month old mice were similarly inoculated intracerebrally (0.03 ml.), subcutaneously (0.3—0.5 ml.) and intraperitoneally (0.5 ml. per animal). One litter was used for each strain. Three weeks later the mice were killed. Sections were prepared from the central nervous system (CNS) and from the body musculature by fixation in 10 % formalin solution, paraffin embedding and staining with haematoxylin and eosin.

Piglets. Hysterectomy was performed on pregnant sows, 3 days before the day of expected delivery. The procedure of *Young et al.* (24, 25) was followed. The operation took place in a room which was solely used for this purpose and which was thoroughly cleansed and disinfected. The personnel were well trained. After the peritoneal cavity had been opened, the uterus was enclosed in a cloth chamber. The piglets were rapidly removed and dried with sterilized towels. The umbilical cords were firmly tied with silk strings. The piglets were then kept individually in metal cages, which had been disinfected with formaldehyde gas. Prewarmed and filtered air was circulated by positive pressure. The temperature in the cages was thermostatically regulated. During the first 3—4 days it was kept at 30°C and during the following week it was gradually lowered to 20°C.

The food consisted of repasteurized cow's milk with one hen's egg per litre, and additions of minerals and vitamins A and D (24, 25). It was prepared under aseptic conditions. Food which had not been consumed after one hour, was removed in order to prevent bacterial growth, which easily occurs at this temperature. The throughs were cleansed and boiled before each feeding.

The criterion of successful raising of the colostrum-deprived piglets, especially during the conditions of the inoculation experiments, was that the non-inoculated controls appeared healthy

and yielded negative results at virological, bacteriological and histological examinations.

After 1 month most of the pigs had grown too big for their cages and the experiments were discontinued.

The pigs were observed through the windows of the cages several times per day. In order to minimize the exposure to external influence, other examinations, such as measuring of the body temperature and counting of blood cells, were omitted.

The virus inoculation of the piglets was made orally on one of the first days of life and not until the animals had started to eat regularly.

Blood samples were taken from the anterior vena cava under light ether narcosis before the inoculation and by intracardial puncture, when the piglets were killed at the end of the experiments.

Faecal specimens were taken with a pair of forceps holding a piece of sterilized gauze from the rectum of the piglets at intervals during the experiments.

At the end of the experiments the pigs were killed by bleeding through intracardial puncture under ether narcosis. Some of the pigs that developed symptoms from the CNS were killed in order to allow isolation of virus from CNS. At the post-mortem examination the CNS was first removed. Specimens for virological examinations were taken under aseptic conditions from the brain stem, cerebellum, medulla oblongata, and the lumbar part of the spinal cord.

In some cases, especially in those pigs that were killed soon after the symptoms had developed, specimens for virological examinations were also taken from the lungs, and for bacteriological examination from the brain stem and the spleen.

The following specimens for histological examination were taken: 5 transversal sections from the brain stem and adjacent parts of the cerebral hemispheres, one comprising the whole cerebellum and one from the medulla oblongata and the cervical, thoracic, and lumbar spinal cord, respectively. Specimens were also taken from the nasal conchae, spleen, liver, lungs, kidneys, heart, and the gluteal musculature. The specimens were prepared as described above for mice.

All materials for virus isolation were stored at —20°C until examined. Specimens from the CNS were suspended in phosphate buffered saline (PBS) to give a 10 % suspension without addition

of ether or antibiotics. The suspensions were left for sedimentation at +4°C for 1 hour.

Specimens from the lungs were suspended in PBS to give a 20 % suspension and centrifuged for 10 minutes at 2.000 r.p.m.

The 10 % faeces suspension in PBS was centrifuged for 10 minutes at 8.000 r.p.m. To suspensions of lungs and faeces antibiotics, 1.000 i.u. of penicillin and 1 μg. streptomycin per ml. were added. The supernatant of all suspensions was inoculated into swine kidney t. c. tubes 0.2 ml. per tube.

At all examinations for the presence of virus in tissues from the pathogenicity tests the first t. c. passage was considered as conclusive if no CPE appeared. Thus, no blind passages were done.

For the bacteriological examinations agar plates with or without addition of 10 % horse blood and 2 % dextrose were used.

Preparation of antisera and sera

Rabbit antisera were produced by 4 weekly intravenous inoculations, each of 3 ml. of virus suspensions, which had been centrifuged for 10 minutes at 2.000 r.p.m. The rabbits were bled 10 days after the last inoculation under ether narcosis. Before being used for immunization each virus strain, which was selected as being representative of a serological group, was purified by the three-fold limit dilution procedure and by the three-fold plaque passage purification. For other viruses (see Table 2) only the limit dilution procedure was used. The production of "pure progeny" of a strain by means of the plaque procedure has been described earlier (20).

All sera used in this work were inactivated at +60°C for 30 minutes and stored at —20°C.

Neutralization tests (NT). The 50 per cent neutralizing endpoints of sera were preliminarily determined in a pre-test so that several sera could be examined at the same time and thus under the same conditions. Sera with low titre and the negative control sera were on some occasions inoculated into swine kidney t. c. tubes without dilution, for checking the absence of virus. For the cross-neutralization test the method of constant amount of virus (mostly 100 TCD_{50}/0.1 ml.) and two-fold serum dilution, was used (17, 18, 19).

To each serum dilution 3 to 5 t. c. tubes were used, each inoculated with 0.2 ml. of the virus-serum mixture. The virus-serum

mixture was kept for an hour at +37°C in a water-bath. The virus suspension containing the coresponding 100 TCD_{50} per 0.1 ml. was further diluted ten-fold and each dilution was inoculated into 5 t. c. tubes, 0.1 ml. into each tube. The tubes were examined for CPE on the 2nd and 3rd day and on the 6th or 7th day, respectively, after the inoculation.

The 50 % neutralizing end-points of the sera calculated by the method of *Kärber,* are given in $\log_{10}$. Unless otherwise indicated, the end-points correspond to 0.1 ml. of the dilution of the serum before the addition of an equal volume of virus suspension and, thus, not to the final dilution (17, 18, 19). As negative serum controls in the NT with swine sera of 4 week old pigs raised without colostrum were used. In NT with rabbit sera the negative serum controls did not neutralize 100 TCD_{50} of the corresponding virus at a final dilution of 1:5 when read 2 to 3 days after the inoculation.

The qualitative neutralization of the isolated strains by rabbit S180/4, S159, and Talfan virus antisera was made as follows. The virus suspension, diluted to contain 100 TCD_{50} per 0.1 ml., was mixed with an equal volume of antiserum. The latter was a twenty-fold concentration of the dilution, which corresponded to the 50 % neutralizing end-points against the homologous virus (0.1 ml. of the antiserum against 100 TCD_{50} per 0.1 ml. of the virus) (8). The virus-serum mixture was as usual kept at 37°C for 60 minutes and then inoculated into 5 t. c. tubes, 0.2 ml. into each tube.

RESULTS

Preliminary serological grouping of the isolates

The results of the serological screening of the isolates in NT using rabbit S180/4, S159, and Talfan virus antisera are shown in Table 1. It will be seen that 5 isolates were neutralized by S180/4 antiserum but only 1 each by S159 and Talfan virus antiserum, at the early reading. Altogether 7, 4 and 3 isolates, respectively, were completely or partially neutralized. For the isolates that proved to be related with either of the prototype viruses, the degree of neutralization of the prototype antisera was determined quantitatively. The 50 % neutralizing end-points of the sera that neutralized the S180/4 virus were lower than the end-point of the homologous antiserum. This was not the case with the strains that were neutralized by the antiserum of S159

Table 1. Neutralization tests with the isolated strains and S180/4, S159, and Talfan rabbit antisera.

Virus strains ($log_{10}TCD_{50}$ 100/0.1 ml.)	Rabbit antisera[1])											
	S 180/4				S 159				Talfan			
	Final dilution 1:25			50 per cent neutralization end-points[2])	Final dilution 1:10			50 per cent neutralization end-points[2])	Final dilution 1:3			50 per cent neutralization end-points[2])
	7	3	7	7	7	3	7	7	7	3	7	7
N5	Rabbit control serum, dilution 1:5 < 0.5	±	+	1.9	Rabbit control serum, dilution 1:5 < 0.5	—	—		Rabbit control serum, dilution 1:5 < 0.5	—	—	
N7		—	—			—	—			—	—	
N8		+	—			+	—			±	—	
N10		+	+	2.3		—	—			—	—	
N11		—	—			—	—			—	—	
U1		—	—			—	—			—	—	
U4		+	+	2.6		—	—			—	—	
U6		—	—			—	—			+	+	2.6
U10		—	—			—	—			—	—	
V4		—	—			—	—			—	—	
V5		—	—			±	—			—	—	
V10		+	+	2.9		—	—			±	—	
V13		±	—			—	—			—	—	
V14		—	—			+	+	2.4		—	—	
V16		+	+	2.5		+	—			±	—	
V18		—	—			—	—			—	—	
S180/4			2.7[3])	3.1			2.1[3])	2.3			2.4[3])	2.5
S159												
Talfan												

[1]) Antisera diluted to contain 20 neutralizing units.

[2]) The quantitative and qualitative determinations of neutralizing antibodies were not made at the same time.

[3]) Homologous 50 per cent neutralizing end-points/0.1 ml. of serum. + = complete neutralization, ± = partial neutralization, — = no neutralization.

and Talfan virus. Strain N8 showed some slight neutralization with all three antisera. Similarly, strain V16, which was neutralized by the S180/4 antiserum, also showed some neutralization with the antisera of S159 and Talfan virus.

Antibody titre of the pigs from which viruses were isolated (Table 2)

The sera of the pigs from which the isolates had been recovered showed a generally fairly low antibody titre against the

Table 2. Neutralization tests with sera of the pigs from which the strains were isolated.

Virus strains ($log_{10}TCD_{50}$ 100/0.1 ml.)	50 per cent neutralization end-points (Swine control serum, dilution 1:5 = < 0.5)		Designation of pig sera
N5		1.6	Ns-5
N7		1.3	Ns-7
N8		< 0.5	Ns-8
N10		1.0	Ns-10
N11		1.7	Ns-11
U1		1.5	Us-1
U4		2.7	Us-4
U6		2.4	Us-6
U10		1.2	Us-10
V4		2.3	Vs-4
V5		0.7	Vs-5
V10		1.4	Vs-10
V13		1.1	Vs-13
V14		1.7	Vs-14
V16		1.0	Vs-16
V18		0.9	Vs-18

own strain. The highest titre reached was 2.7 log_{10} 50 % neutralizing end-point against strain U4. Against 3 strains, N8, V5 and V18, the serum titre of the carriers was < 1.0 50 % neutralizing end-points/0.1 ml. Sera from older pigs showed slightly higher "non-specific" titres (0.5—1.0 neutralizing end-points/0.1 ml.) than sera from 1 month old pigs which had been raised without colostrum.

Strains which were not neutralized by any of the three prototype antisera or partially neutralized by only one of them were compared by cross-neutralization tests (Table 3).

Table 3. Distribution of the 16 isolates according to the results of cross-neutralization tests. The strains in the second column were selected for further investigation.

Serological group	Reference strains	Number of isolated strains	Designation of strains
I.	U6	1	(Teschen), U6
II.	S180/4	5	(S180/4), N5, N10, U4, V10, V16, N8 (?)
III.	S159	1	(S159), V14
IV.	N7	2	N7, V5
V.	N11	1	N11
VI.	U1	1	U1
VII.	U10	1	U10
VIII.	V4	3	V4, V13, V18

Before being inoculated into rabbits for production of antisera all the strains had been treated by the three-fold limit dilution procedure and by the three-fold plaque passage purification. Other strains, *i. e.* N5, N10, V10, V16, V14, V5, V13, and V18, had been treated by the limit dilution method only.

The cytopathogenic effect (CPE) of the strains on t. c. of different species.

The strains selected for further investigation (see Table 3) were inoculated on primary kidney t. c. of dogs, calves, rabbits, guinea pigs, rats, and mice. Eight days after the inoculation no significant changes were seen. In a few cases where a slight CPE was present another passage was done. The CPE did not reappear. Nor did the so-called chich embryo fibroblast cultures, the human kidney t. c. and the human liver t. c. show any CPE.

Pathogenicity for colostrum-deprived piglets.

The results of pathogenicity tests with 6 strains are shown in Table 4. The piglets were mostly inoculated on the 3rd day after the hysterectomy, *i. e.* when they had started to eat regularly and had become fully active. All inoculations were made orally on the assumption that this corresponds to the natural path of the infection. All the inoculated piglets developed disease which was obvious from the symptoms or, in 3 cases, from histological changes only.

Strain N7 produced marked disease. All 3 pigs developed paresis or paralysis of the hind legs 9 to 11 days after the ino-

culation. In pig no. HE-26/3 paresis of the forelegs also appeared after another 3 days. This pig was killed on the same day and virus was recovered from the CNS. No virus was recovered from the CNS in pig no. HE-26/5 which was killed 8 days after the symptoms had appeared. From the lungs no virus was isolated in either of the pigs.

For pigs inoculated with strain N7 the histological changes seem to be most evident in the caudal portions of the CNS (Table 4).

The CPE in the t. c. tubes inoculated with faeces suspension appeared 3 days after the inoculation. The shedding of virus in faeces lasted for at least 11 to 14 days. The finding of 1 positive t. c. tube out of 5 after 30 days in one pig (HE-26/4) indicates that a low-grade shedding was still going on.

Strain U1 also caused obvious neural symptoms. After an incubation period of 8 to 16 days 1 pig showed severe and 2 pigs slight paralysis. As in the pigs inoculated with strain N7 atrophy of the muscles of the hind legs could be seen. In pig HE-26/9 the paretic gait eventually disappeared, and for a few days before being killed, the pig seemed normal.

From pig HE-26/10, which had severe paresis, virus was isolated from the CNS 5 days after the appearance of the symptoms. The histological picture of the lungs was normal. In the other 2 pigs no virus was isolated from the CNS 15 and 13 days, respectively, after the inoculation.

The histological changes in the CNS were most evident in the area of the cervical medulla and the caudal cerebrum. They were especially marked in the basal parts of the cerebrum.

Virus was shed with the faeces for at least 2 weeks in 2 pigs but was still isolated after 29 days in the third pig (HE-26/11).

Two out of 3 pigs inoculated with *strain V4* developed symptoms 10 and 16 days, respectively, after the inoculation. Pig HE-26/7 was able to stand on its hind legs, but when moving it would suddenly sink down into a sitting position. These symptoms eventually became weaker, and before the pig was killed only slight incoordination of the hind legs persisted.

On the 12th and 18th days after inoculation virus was not recovered from the CNS. Histological evidence of a nonpurulent encephalomyelitis was present in all the 3 pigs, most pronounced in the spinal cord.

Virus was isolated from the faeces of all the 3 pigs as late as

Table 4. Results of pathogenicity tests in colostrum-deprived piglets with strains S159, N7, V4, U1, N11, and U10.

Litter No.	Piglets No.	Age at inoculation (days)	Inoculum				Symptoms	Age at killing (days)	Post-mortem changes		Bacterilogical examination	Isolation of viruses from			
			Strain	t. c. passage	neg. $\log_{10}$ TCD_{50}/ml.	Volume and route (ml.)			gross	histological		CNS (days after appearance of symptoms)	Faeces (days after inoculation)		
													0	14	28—30
HE-26	1	1	S159	18	6.1	2p/o	No symptoms	30	—	+	NT	NT	—	+	—
	2	1	S159	18	6.1	2p/o	Moderate paresis of hindlegs 12—18 days p. i.	30	—	+	—	—(18)	—	+	—
	3	1	N7	6	6.5	2p/o	Sitting position 9 days p. i. Paralysis of right foreleg, 12 days p. i.	12	moist appearance of CNS	+	—	+(3)	—	+(11)	——
	4	1	N7	6	6.5	2p/o	Balance disturbances mostly sitting, 11 days p. i. Atrophy of hindleg muscles. During the last week slight improvement of balance.	30	—	+	—	—(19)	—	+	+
	5	1	N7	6	6.5	2p/o	Balance disturbances, mostly sitting, 9 days p. i.	17	moist appearance of CNS	+	—	—(8)	—	+	——

Table 4 (continued).

	6	1	V4	6	6.5	2p/o	Paresis of left hindleg 16 days p. i.	28	—	+	NT	—(12)	—	+	+
	7	1	V4	6	6.5	2p/o	Moderate paresis of hindlegs 10 days p. i.	28	—	+	NT	—(18)	—	+	+
	8	1	V4	6	6.5	2p/o	No symptoms	28	—	+	NT	NT	—	+	+
	10	1	U1	6	6.1	2p/o	Mostly sitting position, nearly unable to stand 8 days p. i.	13	moist appearance of CNS	+	—	+(5)	—	+(13)	+
	11	1	U1	6	6.1	2p/o	Moderate paresis of hindlegs 16 days p. i.	29	—	+	NT	—(13)	—	+	+
	12	not inoculated					No symptoms	30	—	—	—	—	—	—	—
	13	not inoculated					No symptoms	30	—	—	—	—	—	—	—
HE-27	1	3	N11	6	5.7	2p/o	No symptoms	31	—	+	NT	—	—	+	—
	2	3	N11	6	5.7	2p/o	Slight paresis of hindlegs 10 days p. i. and during the following week.	31	—	+	NT	—	—	+	—
	3	3	U10	6	5.9	2p/o	Slight paresis, 8 days p. i.	32	—	+	NT	—	—	+	+
	4	3	U10	6	5.9	2p/o	No symptoms	32	—	+	NT	—	—	+	+
	5	not inoculated					No symptoms	33	—	—	—	—	—	—	—

28 days after inoculation. The CPE in the t. c. tubes inoculated with this material was already apparent 2 days after inoculation.

Strains U10, N11, and S159 caused slight or no symptoms in the inoculated pigs. A moderate weakness in the rear part of the body could be observed in some pigs. It was most evident in the beginning and disappeared gradually in about a week. More or less of histological changes in the CNS could be detected in all pigs.

Strains S159 and N11 were still recovered from faeces after 2 weeks and strain U10 as late as 30 days after inoculation.

The histological changes consisted of isolated or numerous extravascular and perivascular foci of mononuclear cells, microgliosis, and neuronal damage. Among other organs, the lungs of some pigs showed proliferation of mononuclear cells. A more comprehensive description will be published separately.

Serological examinations. The results of the cross-neutralization tests between the isolated strains and strains S180/4, S159, and Talfan, all purified by the three-fold limit dilution method and by the three-fold plaque procedure with rabbit antisera, are shown in Table 5.

Each strain can be distinctly differentiated from other strains and, hence, represents one serological type. There is, however, a marked cross-neutralization between U6 and Talfan. A slight

Table 5. Results of cross-neutralization tests. The strains used had been purified by three plaque passages.

Antisera[1] (rabbit origin)		Virus strains ($log_{10}TCD_{50}100/0.1$ ml.)								
		N7	N11	U1	U6	U10	V4	S180/4	S159	Talfan
N7	(K-311)	2.6	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
N11	(K-175)	< 0.5	2.3	< 0.5	< 0.5	< 0.5	0.7	0.7	< 0.5	< 0.5
U1	(K-313)	0.8	< 0.5	2.3	< 0.5	1.0	< 0.5	< 0.5	1.0	< 0.5
U6	(K-315)	< 0.5	< 0.5	< 0.5	2.2	< 0.5	0.9	0.6	< 0.5	2.0
U10	(K-314)	0.6	0.5	1.3	< 0.5	2.8	< 0.5	0.6	1.0	< 0.5
V4	(K-309)	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	2.6	0.6	< 0.5	< 0.5
S180/4	(K-310)	< 0.5	< 0.5	< 0.5	< 0.5	0.6	0.9	2.5	< 0.5	< 0.5
S159	(K-317)	1.2	< 0.5	0.8	< 0.5	0.6	< 0.5	< 0.5	2.1	< 0.5
Talfan	(K-316)	< 0.5	< 0.5	< 0.5	1.9	< 0.5	< 0.5	< 0.5	< 0.5	2.4

[1]) 50 per cent neutralizing end-points of the pre-inoculation sera < 0.5. Pre-inoculation sera contain less than 50 per cent neutralizing end-points at a dilution 1:5.

Table 6. Neutralization tests with pig sera from the pathogenicity tests.

Serum from pig	Inoculated with strain	Days after inoculation	Age in days	Virus strains ($log_{10}TCD_{50}100/0.1$ ml.)							
				S159	N7	V4	U1	N11	U10	S180/4	U6
HE-26[1])											
1	S159	29	30	(<0.5)[2]) 1.2							
2	S159	29	30	1.6	0.6	<0.5	0.6	<0.5	<0.5	<0.5	<0.5
4	N7	29	30	<0.5	(<0.5)[2]) 2.0	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
6	V4	27	28	<0.5	<0.5	(<0.5)[2]) 2.2	<0.5	<0.5	<0.5	0.7	<0.5
7	V4	27	28			1.6				<0.5	<0.5
8	V4	27	28			1.7					
9	U1	28	29	0.7	0.6	<0.5	(<0.5)[2]) 1.9	<0.5	<0.5	<0.5	<0.5
11	U1	28	29				1.7				
12	not inoc.	—	30	<0.5	(<0.5) <0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
13	not inoc.	—	30	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
HE-27[1])											
1	N11	28	31					2.1			
2	N11	28	31	<0.5	<0.5	<0.5	<0.5	(<0.5)[2]) 1.9	<0.5	<0.5	<0.5
3	U10	29	32	<0.5	<0.5	<0.5	<0.5	<0.5	(<0.5)[2]) 2.1	<0.5	<0.5
4	U10	29	32						1.3		
5	not inoc.	—	33	<0.5	(<0.5) <0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5

[1]) Tests with HE-26 and HE-27 sera were made on different occasions.
[2]) Figures in parentheses refer to pre-inoculation sera.

cross-neutralization is also present between U1, U10, and S159, and between V4 and S180/4. At the early reading, *i. e.* after 2 to 3 days, these relationships between strains are more evident than at the 7 days reading.

Cross-neutralization tests (CNT) with pig sera are shown in Table 6. In comparison with non-inoculated controls, all the inoculated pigs have varying but significant antibody titres against the homologous strains in their sera after 28 to 30 days.

There is no apparent cross-neutralization between the different strains except for S159 and U1, between which there is a slight cross-neutralization. The serological relationship between U1, U10, and S159, which was evident at the CNT with rabbit antiserum, was not confirmed by the use of pig sera.

As has already been mentioned, the piglets were inoculated with strains which had been purified by the limit dilution method. The rabbit antisera, used in the CNT were also purified by the three-fold plaque procedure. Passages of strains N7 and U1, which had been purified by either of the methods, were therefore compared (Table 7). As they produced the most marked symptoms from the CNS, these strains were also compared with strains S180/4 and U6, which also produce CNS disturbances. No differences can be seen between passages of N7 and U1, respectively, which had been treated by the limit dilution and plaque passages. The serological differences between these strains and S180/4 and U6 are evident, however.

DISCUSSION

Field diseases in pigs, which are caused by enteroviruses are still incompletely known. As a first step in solving such problems the isolation and characterization of enterovirus strains is well justified.

It appears from the results of the present work and from an investigation in the autumn of 1960 (20) that enteroviruses have a fairly large distribution among pigs in the central part of Sweden. Results of serological and virological examinations which will be published later, indicate similar conditions in other regions of the country as well.

With regard to the geographical distribution of the isolated strains, no definite conclusions can be drawn. S180/4 is most frequent in all the three slaughter house materials. The question whether the accumulation of V4-related strains in the Västerås

Table 7. Neutralization tests with rabbit antisera and strains N7, U1, S180/4, and U6. Of strains and U1 both limit dilution and plaque purified passages were used.

Antisera		Viruses											
		N7				U1				S180/4		U6	
		1. d.[1]		pl.[1]		1. d		pl.		pl.		pl.	
		$Log_{10}TCD_{50}$/0.1 ml. (100)											
		Results of readings after days											
		3	7	3	7	3	7	3	7	3	7	3	7
N7	a[2])	2.5	2.3	2.6	2.3	NT	NT	NT	NT	0.6	<0.5	<0.5	<0.5
	b[3])	>3.2	2.6	>3.2	2.6	NT	NT	1.0	<0.5	0.8	<0.5	0.7	<0.5
U1	a	NT	NT	NT	NT	2.8	2.4	3.1	2.6	<0.5	<0.5	0.6	<0.5
	b	NT	NT	1.1	<0.5	2.9	2.5	2.9	2.6	0.6	<0.5	0.6	<0.5
S180/4	b	<0.5	<0.5	0.6	<0.5	<0.5	<0.5	0.7	<0.5	2.9	2.6	NT	NT
U6	b	<0.5	<0.5	0.8	<0.5	<0.5	<0.5	0.6	<0.5	NT	NT	2.5	2.3

[1]) l.d. = three-fold limit dilution purified; pl. = three-fold plaque purified in addition.
[2]) a = antisera produced with three-fold limit dilution purified strains.
[3]) b = antisera produced with three-fold limit dilution and plaque purified strains.

material represents a tendency or is merely a random effect cannot be answered at present.

Ether-resistant viral agents were isolated in 20 to 35 per cent of the examined faecal specimens. Similar reports have been published from other countries (2, 5, 6, 7, 22, 23).

The isolates examined in the present study have all been designated as enteroviruses. Terms like ECPO and ECSO have been avoided, because knowledge of the properties and the epizootology of the swine enteroviruses is still fairly meagre. The name "orphan" originates from the designation of human enteroviruses. Their disease-producing capacity was unknown at the time they were isolated but has later become known for some of them.

Five out of the 16 ether-resistant isolates belong to the serological group S180/4. Two of them originate from the area of Nyköping, 2 from Västerås and 1 from Uppsala. Strain S159 (S = Stockholm) was also isolated from the area of Västerås. Examination of field sera from swine (to be published) often shows neutralization titres against S180/4 and S159. Strains which are serologically identical with or closely related with S180/4 have been isolated in Germany (6) and England (18).

One of the isolates, V14, was neutralized by the S159 and another, U6, by the Talfan rabbit antiserum. However, sera from slaughter pigs fairly often had neutralizing titres against Talfan virus (19), which would indicate a more frequent occurrence of immunologically related viruses. Yet, it is hardly possible to draw general conclusions concerning the distribution of enteroviruses from a small number of specimens, which were collected during a limited period of time. At any rate, the occurrence of the strains in question as well as of antibodies in the pigs has been confirmed.

In characterizing the selected strains as enteroviruses their resistance to ether was demonstrated. In the following examination the procedure recommended by the Committee on the Enteroviruses was used (8). As there are so far no generally accepted criteria for swine enteroviruses, one representative of each serological group was also tested for resistance to chloroform. Further, the CPE in swine kidney t. c. and the long duration of elimination via the faeces were demonstrated. For all the 8 selected strains the particle size was less than 30 mμ, as evident from the sedimentation constants (6, 14, 21). These properties

also correspond to those which have been tentatively proposed by *Betts et al.* (5) and partly to those reported by *Bögel & Mayr* (6).

Determination of the particle size by filtration or by gradient centrifugation is generally accepted to be of great importance in the classification of enteroviruses (1). The adenoviruses, which are also resitant to ether, differ from the enteroviruses by their larger size (about 70 $m\mu$). Whereas the enteroviruses contain ribonucleic acid (RNA) the adenoviruses contain deoxyribonucleic acid (DNA). The reoviruses (ECHO 10) are resistant to ether and contain RNA but are approximately of the same size as the adenoviruses (16). Neither the adenoviruses nor the reoviruses have so far been demonstrated in pigs, although it is possible that they occur in this species. It is known, however, that several adenoviruses, *e. g.* types 1—7 of the human strains (9) and the virus of the contagious viral hepatitis in dogs, produce a CPE on swine t. c.

The reoviruses have a broader species spectrum with regard to the CPE than have the enteroviruses. The strains, that are described in this paper, produced a CPE only in swine t. c. but not in t. c. of other species examined. They are not pathogenic to suckling mice. No publications on the pathogenicity of adenoviruses and reoviruses to colostrum-deprived piglets have as yet appeared.

The antibody contents of sera of pigs from which the enterovirus strains were isolated vary between low and high, i. e. 0.5—2.7 $\log_{10}TCD_{50}/0.1$ ml. *Mayr* (12) showed that after oral inoculation of Teschen virus in pigs, antibodies could be demonstrated within 1 week and then reached a significantly high titre of long duration. This was also true of pigs which did not fall ill. The relatively low antibody titres in some pigs from which enteroviruses could be isolated may indicate that these pigs were in an early stage of infection.

The examined strains produced pathogenic effect in colostrum-deprived piglets. This was especially noticeable with strains N7 and U1, which caused severe paresis and histological changes in the form of non-purulent encephalomyelitis. The latter strains were also isolated from the CNS of inoculated pigs.

The use of colostrum-deprived piglets is a sensitive method for pathogenicity tests with swine viruses. Because of the epithelio-chorial type of the swine placenta, the foetuses are con-

sidered to be protected from infections and, further do not acquire antibodies from the circulating blood of the sow. The principles of raising piglets without the use of colostrum are simple. No difficult problems arise when experience has been gained and the personnel is well trained. The losses are then limited to single piglets, which are shocked because they are not promptly removed from the uterus, and to piglets that are underdeveloped or misshaped. Out of 19 piglets which were used for pathogenecity tests, only 1 died. The cause was suffocation at the hysterectomy. Further, the non-inoculated control piglets showed no post-mortem changes, including histological findings, and the bacteriological and virological examinations were negative.

Colostrum-deprived pigs, raised on cow's milk and eggs, developed mature serum-protein profile at the age of 3 weeks, whereas pigs had been feeding from the sow, matured at 2 weeks (11). In the present work all colostrum-deprived pigs which had been inoculated with enterovirus during one of the first 3 days of life showed significant neutralization titre at 1 month. In the test with strain U6 some titre was evident even after 2 weeks. These observations are in agreement with those of *Beran et al.* (3).

The oral route of inoculation was used at the pathogenicity tests, since it can be supposed to be natural path of entrance into the body. That a faecal elimination also occurs after intracerebral inoculation has been demonstrated for strain U6 (19). Similar observations were made by *Betts and Jennings* (4), who used the enterovirus strains T80 and T52A.

Low passages, *i. e.* not higher than the 6th, were used for most strains in the pathogenicity tests. That passages in t. c. exert a modifying action on the pathogenic properties of a virus has been pointed out repeatedly. The Teschen strain Konratice, after numerous swine kidney t. c. passages, showed a marked weakening of the virulence, as well as changes of its physical-chemical properties (14). *Moscovici et al.* (15) possibly noted modification of bovine enteroviruses which were passaged in t. c. Decrease or loss of virulence after numerous t. c. passages have been demonstrated for most viruses. In the present work strain S159, of which the 22nd t. c. passage was used, produced the weakest symptoms and histological changes. Whether this was a consequence of the many preceding passages could not be determined, because less passaged virus material was not available. Further, the number of inoculated piglets was small.

Strains N7 and U1 produced a severe disease in the experimental pigs. These strains are characterized by rapidly developing, large and distinctly defined plaques. Strain S159, under the same conditions, also showed this type of plaques, although it possessed weak pathogenic properties. Strain U6 whose pathogenicity is similar to that of N7 and U1, produced small plaques. It is therefore hardly possible to find a correlation between virulence and the shape of the plaques produced.

The difference between strain U6, which belongs to the Teschen group, and strain S180/4 on one hand, and the strains examined in this work on the other, is demonstrated serologically.

Limit dilution and plaque purified passages of the strain which proved to be the most virulent ones in the pathogenicity tests N7 and U1 were compared in cross-neutralization tests. There was no difference between the passages, irrespective of the method of purification, which indicates that a mixture was not present. This is of special interest with regard to S180/4 and U6, which produce a similar pathogenic effect (17, 19).

The pathogenicity tests on colostrum-deprived pigs clearly show the facultative pathogenic properties of the examined strains and their neuro- and enterotropic character. As demonstrated for strain U6 in pigs which had received colostrum, the passive immunity is of great importance for the virulence. From investigations with serum-protein fractions of pooled slaughter swine sera, which are not yet completed, it appears that the γ-globulin fraction neutralizes all the strains which have been examined in this work. The sera used for preparation had been collected from the Uppsala area. I am indebted to Dr. *Flodin,* Pharmacia, Uppsala for preparation of the serum fractions.

In tests with rabbit sera cross-neutralization was present between U1, U10, and S159 and between V4, and S180/4, respectively. With swine serum the same was demonstrated only between U1 and S159 and between V4 and S180/4. The true nature of this is not yet clear.

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SUMMARY

Sixteen isolates of ether-resistant viruses from pigs in the central part of Sweden were compared with known swineenterovirus prototypes by neutralization tests. Altogether 8 different serological groups were obtained. One of them belongs to the Teschen group. One representative of each group was selected for tests for pathogenicity to experimental animals, and cytopathogenicity to tissue culture of different species.

All strains produced paresis and histological evidence of encephalomyelitis when inoculated orally, on 1—3 day old piglets which had been obtained by hysterectomy and were raised without colostrum. Virus was recovered from the faeces of inoculated piglets for at least 14 days p. i.

New-born mice and adult mice proved to be resistant as judged from absence of symptoms and histological changes. Cytopathogenic changes were produced in tissue cultures from pigs but not from mice, guinea-pigs, dogs, calves, and man.

The serological grouping was made by neutralization tests using rabbit sera and sera from the experimental piglets. All viruses used for

production of rabbit antisera had been purified by the limit dilution and consecutive plaque methods.

The described viruses are characterized as swine enteroviruses because they are cytopathogenic to swine tissue culture, resistant to ether, eliminated via the faeces for a rather long time, and have a sedimentation constant of the same order of size as Teschen control-virus.

ZUSAMMENFASSUNG

Untersuchungen und Charakterisation der Enterovirusstämme bei schwedischer Schweinen. II. Pathogenitätsversuche und serologische Eigenschaften.

Sechzehn ätherresistente Stämme von Schweinen aus Mittel-Schweden wurden mit bekannten Schweinenenteroviren mittels Neutralisationstest verglichen. Es wurden ingesamt acht verschiedene serologische Gruppen ermittelt, wovon eine den Viren der Teschener Gruppe gehört. Je ein Repräsentant der einzelnen Gruppen wurde gewählt für die Prüfung der Pathogenität an Versuchstieren, als auch für die Cytopathogenität in Gewebekulturen von verschiedenen Tierarten.

Alle Stämme die peroral den 1—3 Tage alten, durch Hysterektomie erhaltenen und ohne Kolostrum aufgezogenen Ferkeln gegeben wurden, verursachten bei diesen eine Parese und ergaben histologisch eine Encephalomyelitis. Aus Kot der geimpften Ferkeln wurde das Virus isoliert mindestens 14 Tage nach der Infektion.

Neugeborene oder erwachsene Mäuse erwiesen sich gegenüber den isolierten Stämmen als resistent, wie es durch die Abweseheit der Symptomen und histologischen Veränderungen zu entnehmen wäre. Die cytopathogene Veränderungen entstanden in Schweinegewebekulturen, jedoch nicht in Mäuse-, Meerschweinchen-, Hunde-, Kälber- und Menschengewebekulturen.

Die serologische Gruppierung wurde in Neutralisationsversuchen mit Kaninchenseren und Seren der Versuchsferkeln, durchgefürt. Alle für die Herstellung von Kaninschenantiseren angewendete Stämme wurden mit dem Verfahren der dreimaliger Endverdünnung und darauffolgendem Plaque-Verfahren, unterzogen.

Da die beschriebene Viren Eigenschaften wie Cytopathogenität für Schweinegewebekultur, relative Resistenz gegenüber Äther, eine längere Ausscheidungsdauer durch Kot und eine ähnliche Sedimentationskonstante als das Virus der Teschener Krankheit haben, sind diese als Schweineenteroviren charakterisiert.

SAMMANFATTNING

Undersökningar och karakteristik av enterovirusstammar hos svenska grisar. II. Patogenitetsförsök och serologiska egenskaper.

Sexton isolat av eterresistenta virus från grisar i Mellan-Sverige jämfördes genom neutralisationstester med kända prototyper av svinenterovirus. Åtta serologiskt skilda grupper erhölls. En av dem tillhörde Teschengruppen. En representant för varje grupp utvaldes för

undersökningar på patogenitet för försökssvin och på cytopatogenitet i vävnadskultur från olika species.

Alla stammarna orsakade pares samt histologiska förändringar typiska för encephalomyelit, när de ympades peroralt på 1—3 dagar gamla grisar, vilka hade erhållits genom hysterektomi och uppföddes utan suggmjölk. Virus kunde isoleras från faeces av ympade grisar under minst 14 dagar.

Nyfödda respektive adulta möss visade sig vara resistenta i det de inte visade några symptom eller histologiska förändringar.

Cytopatogena förändringar erhölls i vävnadskultur av svin men inte av möss, marsvin, hundar, kalvar och människa.

För neutralisationstesterna användes sera av kaniner och försökssvin. Alla virus, vilka användes för framställning av kaninantisera hade renats genom *limit dilution* och konsekutivt plaque-förfarande.

De beskrivna virus karakteriseras som enterovirus, emedan de är cytopatogena för svinvävnadskultur, resistenta mot eter, elimineras med faeces under relativt lång tid och har en sedimentationskonstant av samma storleksordning som virus tillhörande Teschen-gruppen.

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