SEROLOGICAL AND BIOLOGICAL STUDIES ON PORCINE ENTEROVIRUSES ISOLATED IN CANADA

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Introduction and Literature Review

The determination of porcine enterovirus relationships has been of importance since it was demonstrated that many of the non-pathogenic strains are biologically and physically similar to the pathogenic strains which produce Teschen and Talfan disease (3, 12, 19). This study was undertaken to compare nine strains of porcine enterovirus isolated in Canada to the ECPO6 strain isolated in Ohio (21), the T80 strain isolated in England (2), and Talfan disease virus (5).

Comprehensive reviews have been published covering the isolation and the biological and serological characterization of the porcine enteroviruses (8, 14, 16, 19 and 23). Table I summarizes the results of direct comparative serological studies on porcine enteroviruses from worldwide sources with the type designations based on the original classification scheme proposed by Betts *et al.* (3) in 1961. These workers suggested four criteria for the classification of a virus among the porcine enteroviruses: (1) size of the order of 30 to 40 millimicrons, (2) ether resistance, (3) enterotropism and recovery from feces for sufficiently long periods to provide evidence of multiplication, and (4) cytopathogenicity on porcine kidney cell cultures.

The known porcine enterovirus strains can be divided into two groups by the cytopathic effect they produce in porcine kidney cell cultures (7, 9, 10, 13, 19, 22 and 24). The majority of these produce a cytopathic effect which is characterized by the appearance of clumps of enlarged, rounded, refractile cells. The early foci extend peripherally to involve the whole sheet. Some cytoplasmic strands are observed and these become more evident as an increasing number of cells are involved. This type of cytopathic effect was first described by Betts (2) for the T80 strain he isolated. The other enteroviruses produce an effect in porcine kidney cell cultures which is characterized by the random appearance of slightly enlarged, rounded cells, and by increased granularity of the cell cytoplasm. The affected cells appear rosette-like due to the formation at the periphery of cytoplasmic protrusions through the cell membrane with the formation of prominent cytoplasmic strands. Lamont and Betts (11) first described this type of cytopathic effect for the V13 strain they isolated. For the sake of convenience they will be referred to as Type I and II, respectively.

Materials and Methods

Viruses

Eight of the isolates studied (PE1, 3, 4, 5, 6, 7, 8, 10) were recovered from the brain or feces, or both, of diseased pigs or from the feces of healthy pigs. Strain PEA was recovered from a hemolyzed blood sample obtained from a pig infected with a virulent strain of hog cholera virus. The strains had been passaged from two to six times in cell cultures prior to their use in this trial. The isolates were submitted to a purification procedure which consisted of three serial, terminal dilution transfers in pig kidney cell cultures. The three foreign strains[1] were not submitted to the purification process but were handled as they had been received from the workers who isolated them. Following the purification process, a pool of each of the 12 viruses was produced, by inoculating several pig kidney cell cultures with the viruses and harvesting these at the time of maximum cytopathic effect. The harvested virus suspensions were frozen

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[1]The ECPO6 strain was obtained from Dr. E. H. Bohl, Ohio State University, Columbus, Ohio; the T80 strain was obtained from Dr. A. O. Betts, of the Cambridge Veterinary School, Cambridge, England, and the strain of Talfan virus was obtained from Dr. J. T. Done, Central Veterinary Laboratory, Weybridge, Surrey, England.

TABLE I

CLASSIFICATION OF PORCINE ENTEROVIRUS SEROTYPES ACCORDING TO THE ORIGINAL STRAIN DESIGNATION PROPOSED BY BETTS *et al.* (3)[1]

Serotype	Reference							
	Betts *et al.* (3)	Bogel and Mayr (4)	Huck *et al.* (8)	Morimoto and Watanabe (14)	Pette (15)	Sibalin (20)	Szent-Ivanyi (16)	Rasmussen (16)
I	Teschen Talfan		E1	D4, SF12, SFK2		U6	5D, 7G	Da1
II	T80-T52A	Tubingen 1	E4	SFK10	Mun.5	180/4	12PL-Riems 52	Da2
III	F7							
IV	V13			SFH1, 2 and 6 D28, S3, SF16				
V	F12							
VI	F34							
VII	F70							
VIII	F76							

[1]Only those strains which had been compared in direct cross-neutralization test have been included. Designations are those used in the original publication.

and thawed once, pooled and centrifuged at 500 G to deposit cell debris. Clear supernatant fluids were then considered as the pooled virus suspension, and were dispensed into glass ampoules and frozen either in a mechanical freezer at −20° C. or in a dry-ice in alcohol bath at approximately −65° C.

Antiserum Preparation

Four young-adult rabbits were immunized against each virus strain using a series of five daily intravenous injections of 1 ml. of the pooled cell culture fluids. This series of injections was followed one week later by a single dose of the virus suspension. Approximately 10 days after the last inoculation the rabbits were bled out, the serum harvested, pooled, filter sterilized and heated to 56° C. for 30 minutes. All antisera were frozen when stored. Preinoculation blood samples were collected and harvested serum was pooled and treated in the same manner.

Virus Titration

Replicate titrations were carried out in pig kidney cell cultures on all of the viruses in order to determine accurately the virus titer of each pooled sample. The infective virus titers were estimated by the method of Reed and Muench (17) in tissue culture infective doses 50 per 0.1 ml. (TCID 50/0.1 ml.), that is the amount of virus in 0.1 ml. of diluted virus suspension capable of infecting 50% of the cell cultures inoculated. The method of preparation and media used in the pig kidney cell cultures have been described previously (22). Prior to use in neutralization tests the virus suspensions were diluted to contain a final concentration of 100 TCID 50/0.1 ml.

Neutralization Tests

Using the antisera and purified virus strains, cross neutralization tests were carried out, by the constant virus varying antiserum technique (18). Serial ten-fold serum dilutions were made in phosphate buffer solution and mixed with equal volumes of the virus suspension containing an estimated 100 $TCID_{50}$/0.1 ml. The serum dilution series ranged from a final dilution of 1:2 to 1:200,000. Serum-virus mixtures were incubated for two hours at room temperature. A 0.2 ml. amount of the incubated serum virus mixture was added to each of four pig kidney cell culture tubes. The further dilution of the serum-virus mixture in the cell culture maintenance medium, was not taken into account in calculating the total serum dilution. Parallel titrations were carried out on the virus suspension and 0.1 ml. of diluted virus was inoculated into each of four pig kidney cell culture tubes. The inoculated cell cultures were examined on the third and on the fifth day post-inoculation and were considered positive if any definite cytopathic effect was detectable. Titer ratios as proposed by Archetti and Horsfall (1) were used to compare the cross-neutralization test results between the viruses studied. Comparison of the results of cross neutralization tests by means of titer ratios provides an objective evaluation of the results of these tests. The titer ratio (R) is the geometric mean of the heterologous serum neutralization titer over the homologous serum neutralization titer for each one of the two viruses being compared. In this way the titer ratio takes into account a total of four values. By definition the homologous titer ratio is one and identity between two virus strains would be represented by values of 1/R equal to or approaching one.

Cytopathic Effects

Cytopathic effects were evaluated by examining affected cell culture tubes with a light microscope at regular intervals during the development of lesions. This was found to be preferable to the use of the fixed and stained preparations, at least where type classification of the cytopathic effect was concerned.

Ether Resistance

Four parts of clear, virus infected cell culture fluids, were mixed with one part ethyl ether (anesthetic grade) and stored for 18 hours at 4° C. The excess ether was removed by evaporation at 37° C. for 30 minutes. An untreated control aliquot was handled in a similar manner and both portions were titrated in pig kidney cell cultures using a ten-fold dilution series.

Results

The cross-neutralization titers obtained for the 12 porcine enteroviruses are presented in Table II. The mean virus dose

TABLE II

RECIPROCAL CROSS NEUTRALIZATION TITERS OF 12 PORCINE ENTEROVIRUSES

Virus	Antiserum											
	PE1	PE3	PE4	PE5	PE6	PE7	PE8	PE10	PEA	T80	ECPO6	TALFAN
PE1	6324[1] (111)[2]	....	<2	<2	43	200	43	113	340	....	431	431
PE1	9282 (76)	928	....	....	....	....	....	....	....	6324	....	....
PE3	20000	9282 (233)	<2	<2	20	63	43	431	2000	632	200	....
PE3	....	6324 (100)	....	....	....	....	....	....	....	....	....	431
PE4	9	4	4309 (137)	928	20	928	20	63	6	6	6	2
PE5	<2	<2	200	431 (105)	<2	338	<2	<2	<2	<2	<2	<2
PE6	2	<2	<2	<2	93 (38)	<2	<2	<2	<2	<2	<2	....
PE6	....	....	....	....	293 (138)	....	....	....	....	....	....	63
PE7	<2	<2	431	928	<2	4309 (155)	<2	2	<2	<2	<2	<2
PE8	9	2	<2	<2	6	<2	702 (56)	4	....	....	<2	....
PE8	....	....	....	....	....	....	63 (105)	....	6	4	....	2
PE10	93	928	<2	<2	4	9	4	928 (47)	632	93	43	<2
PEA	632	63	<2	<2	<2	4	6	632	928 (117)	20	200	<2
T80	<2	63	<2	<2	<2	<2	2	<2	<2	115 (190)	<2	<2
ECPO6	33	200	<2	<2	4	9	6	93	632	93	93 (200)	ND
TALFAN	20	12	2	2	9	4	6	2	<2	<2	20	9282 (165)

[1]Represents the reciprocal of the 50% neutralization titer, in this case of PE1 antiserum against PE1 virus.
[2]TCID 50/0.1 ml. used in all the tests in the same line.
.... = Not tested.

used in the neutralization tests was 129 $TCID_{50}/0.1$ ml with a range of 38–233. The preinoculation rabbit sera did not neutralize any of the 12 enteroviruses to a detectable level. The titer ratios calculated from the reciprocal cross-neutralization titers are given in Table III. Several broad groupings can be distinguished through an examination of the titer ratios. One group includes viruses PE1, PE3, PE10, PEA, T80, and ECPO6. A second group is made up of viruses PE4, PE5. and PE7. Viruses PE6, PE8, and Talfan virus did not react to significant levels with any of the other enteroviruses.

Table IV summarizes the results of examination of various viruses for ether resistance and type of cytopathic effect. All 12 viruses were ether resistant. Nine of the porcine enteroviruses produced a type I cytopathic effect; the other three enteroviruses produced the type II cytopathic effect.

Discussion

Study of the 12 porcine enteroviruses has permitted them to be classified into five sero-groups by means of the serum neutralization test. It is of importance to determine where possible, the relationship of these sero-groups to the sero-group classification proposed originally by Betts *et al.* (3). The results reported herein allow the authors to classify strains PE1, PE3, PE10, PEA, and ECPO6 in the group II porcine enteroviruses along with the 10 strains indicated in Table I. Talfan virus, of course, is included as a sub-type of the group I enteroviruses. Porcine enteroviruses strains PE4, PE5, and PE7

TABLE III

Results of Cross-Neutralization Tests Expressed as Titer Ratios

	PE1	PE3	PE4	PE5	PE6	PE7	PE8	PE10	PEA	T80
TALFAN	83[1]	345	333	1429	250	3333	250	2000	333	1000
ECPO6	6.3	4.5	286	200	143	200	100	5	0.83	11
T80	13	5.3	303	222	111	1000	100	33	100	
PEA	5.3	8.3	833	625	303	1000	40	1.4		
PE10	25	4.8	250	625	143	500	200			
PE8	111	250	385	555	111	1666				
PE7	370	833	7.1	2.5	625					
PE6	83	200	143	200						
PE5	1667	2000	3.1							
PE4	1667	3333								
PE3	2.2									

[1]Titer ratio expressed as 1/r.

TABLE IV

Ether Sensitivity Trials and Type of Cytopathic Effect

	Ether Sensitivity		Cytopathic Effect
Virus	Treated	Untreated	Type
PE1	316[1]	41	I
PE3	316	414	I
PE4	10000	3162	II
PE5	414	414	II
PE6	10	32	I
PE7	100	215	II
PE8	100	41	I
PE10	6761	41410	I
PEA	32	32	I
T80	4.6	3.2	I
ECPO6	ND	ND	I
TALFAN	ND	ND	I

[1]$TCID_{50}$ per 0.1 ml.

form a homogeneous group as far as serological classification and cytopathic effect is concerned. The authors have found that all of the porcine enteroviruses producing the type II cytopathic effect were related antigenically and may in this light fit into the sero group IV which now includes the V13 strain. No evaluation of the position of strains PE6 and PE8 is possible until these strains have been compared with some of the other porcine enteroviruses isolated elsewhere. It would appear that some antigenic variation occurs between the members of serogroup II porcine enteroviruses. This variation is indicated by the titer ratios found in comparisons of the various members of this group. Nonreciprocal neutralization between strains PE1 and T80 have been reported previously by Greig *et al.* (6) and is reflected here by a titer ratio of 13, indicating a slight antigen relationship. Strain PE1 is included in the sero-group II enteroviruses due to its close relationship to strains PE3, PEA, and ECPO6. It was pointed out by Mayr (12) that the porcine enteroviruses fitted into several serotypes with numerous sub-types.

The variation in actual virus doses used in the neutralization tests appeared to be due to variations, however minor, in the quality of the cell cultures used. Because of this, homologous neutralization tests were included at all times to allow direct comparisons.

Ether resistance is one of the characteristics required of a porcine virus for inclusion within the enterovirus group. All of the strains examined by us proved to be ether resistant. Cytopathic effect has proven to be a stable characteristic in our studies of the porcine enterovirus strains and was, as reported by Hahnefeld (7), serotype specific at least between the cytopathic types described.

Summary

Twelve porcine enterovirus strains were found by serum cross-neutralization tests in porcine kidney cell cultures, to belong to five distinct sero-groups. These sero-groups have been related, where possible, to the sero-groups proposed in the literature. All 12 porcine enterovirus strains were ether resistant. Nine of the viruses produced one type of cytopathic effect and the remaining three viruses produced a second type of cytopathic effect in porcine kidney cell cultures.

Résumé

L'étude de 12 entérovirus porcins a permis de les classifier en cinq groupes sérologiques distincts. Les comparaisons furent faites en cultures cellulaires de rein de porc, par la méthode des neutralisations croisées entre virus et antisérums de lapins. Les résultats obtenus ont permis de classifier certains de ces entérovirus porcins dans les groupements sérologiques formulés dans la littérature à ce sujet. Tous les virus étudiés résistèrent aux effets de l'éthyl éther pour une période de 18 heures à 4° C. Neuf des entérovirus, formant quatre des sérogroupes, produisirent un type d'effet cytopathogène en cultures cellulaires et les trois autres, formant le cinquième sérogroupe, produisirent un second type d'effet cytopathogène.

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ERRATUM

Can. Vet. J. 7: 93. 1966.

Page 94, line 7, should read "Duncan McEachran, who had graduated from the Edinburgh Veterinary School under William Dick, had become a disciple of Gamgee who was a veterinarian ahead of his time in the field of infectious diseases and who was to become the father of the International Veterinary Congress" rather than "Duncan McEachran, who had graduated from the Edinburgh Veterinary School under William Dick but had become a disciple of Gamgee, was a veterinarian ahead of his time in the field of infectious diseases and was to become the father of the International Veterinary Congress".