SCREENING OF PIG SERA FOR ANTIBODIES TO JAPANESE ENCEPHALITIS VIRUS USING A DOT ENZYME IMMUNOASSAY AND IgM CAPTURE ELISA: COMPARISON WITH THE HEMAGGLUTINATION INHIBITION AND PLAQUE REDUCTION NEUTRALIZATION TESTS

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Abstract. A dot enzyme immunoassay for determination of antibodies to Japanese encephalitis virus was designed for use as a field technique for the surveillance of Japanese encephalitis virus activity among domestic pigs. The test was compared with the neutralization test and the hemagglutination inhibition test and found to be more sensitive than the hemagglutination inhibition test and comparable to the neutralization test in sensitivity but more simple to perform than either the neutralization or the hemagglutination inhibition tests. An IgM capture ELISA for the determination of JEV specific porcine IgM was also utilized to determine current infection rates in pigs. The tests which do not involve the determination of specific IgM are better used for testing sentinel animals for providing clues as to the rate of transmission of JEV among pigs. IgM tests determining acute infection are less likely to be useful unless animals are tested very frequently or if a great number of animals are tested at any one time.

INTRODUCTION

Japanese encephalitis virus (JEV) is a mosquito borne virus of the family Flaviviridae. It is endemic in many parts of Asia including tropical and subtropical areas. Although much has been achieved in controlling the disease in Taiwan and Japan, severe epidemics of viral encephalitis caused by JEV still occur in some of the less developed parts of Asia such as Nepal, Burma and northern Thailand.

Japanese encephalitis (JE) is a zoonotic disease, only incidentally affecting humans when there is high virus transmission among the animal reservoir, principally pigs. It has thus been a practical policy in some countries to monitor seroconversion in sentinel animals such as pigs or chickens, as part of their JE surveillance. Apart from monitoring sentinel animals, it is also possible to monitor the rate of transmission of JEV by determining the presence of JEV antibody positive animals (generally pigs) either at selected farms or at the point of slaughter.

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The serological test of choice in these monitoring systems has been the hemagglutination inhibition (HI) test (Clarke and Casals, 1958) which employs goose erythrocytes and viral hemagglutinins prepared from sucrose acetone extracted infected suckling mouse brain. This study examines the use of a nitrocellulose membrane based dot enzyme immunoassay (DEIA) as an alternative method to the hemagglutination inhibition (HI) test for the determination of antibodies against JEV. At the same time, an ELISA to determine the presence of JEV specific porcine IgM has also been tested.

MATERIALS AND METHODS

Viruses

Viruses used were Japanese encephalitis virus (JEV), Nakayama strain, and dengue 2 virus (DEN2) strain 16681, both generous gifts of Dr James Porterfield of the University of Oxford. These were propagated in the *Aedes albopictus* cell line C6/36 grown in Leibovitz 15 medium containing 1% heat inactivated fetal bovine serum.

Specimens

Blood was collected from pigs at the point of

slaughter and allowed to clot. The pigs were normally 6 to 9 months of age when brought for slaughter. Serum separated was stored at -40°C until use. One batch of samples were initially collected in March 1989 for a preliminary study. Subsequently additional samples were collected in December 1989 for further study.

Hemagglutination inhibition test (HI)

This was performed essentially as described by Clarke and Casals (1958).

Dot enzyme immunoassay (DEIA)

This has been previously described in detail for dengue 3 virus (Cardosa *et al,* 1988). In this study, JEV antigens prepared from JEV infected C6/36 cells were used (Cardosa *et al,* 1991). The virus antigens and uninfected controls were spotted onto nitrocellulose filters, dried and blocked with phosphate buffered saline (PBS) containing 5% non-fat skimmed milk. These filters were stored dry at 4°C until use. Presence of antibodies to JEV was determined by incubating filters with serum dilutions for 60 minutes at room temperature, washing with PBS and then incubating with protein A conjugated with horseradish peroxidase (KPL, USA) for another 60 minutes at room temperature. The diluent used was PBS containing 5% non-fat skimmed milk. Antibody bound was visualized using 4-chloro-1-naphthol and hydrogen peroxide.

Plaque reduction neutralization test (PRNT)

Porcine fibroblast (PS Clone D) cells were used in an assay employing 24 well cluster dishes and a carboxymethyl cellulose overlay. 100 µl of virus dilution was mixed with 100 µl of serum (heat inactivated at 56°C for 30 minutes) dilution and incubated for 60 minutes at 37°C, after which a suspension of PS Clone D cells in Leibovitz 15 medium containing 1% fetal bovine serum was added to give 1×10^5 cells per well. The plates were incubated at 37°C for 2 to 3 hours until cells had adhered to the plastic, and carboxymethyl cellulose was added to a final concentration of 0.75%.

After incubation at 37°C for 3 to 5 days, monolayers were stained with naphthalene black, and plaques counted. The reciprocal endpoint titer at which there was at least 50% of inhibition of plaques was recorded.

IgM capture ELISA

The assay used was similar to that described by Bundo *et al* (1986) and performed essentially as described in Cardosa *et al* (1991) using microtiter plates (Nunc, Denmark) coated with anti-human µ chain which is known to cross react with porcine µ chain (Dako Cat No. A426 from Dakopatts, Denmark). Pig sera in duplicate dilutions of 1 : 100 were reacted with the anti µ antibodies overnight at 4°C, after which the wells were washed and JE or control antigens added for further 2 hour incubation at room temperature. After washing again, the antigen bound was reacted with a hyperimmune human anti-flavivirus IgG affinity purified and conjugated with horseradish peroxidase. Conjugate bound was visualized using o-phenylenediamine and hydrogen peroxide and optical density determined at 492 nm. The ratio of the OD of Antigen to Control Antigen (Ag/C) was determined and a cutoff ratio of 2 was used to determine positivity in this study.

RESULTS AND DISCUSSION

Ninety-four sera initially collected in March 1989 were tested by PRNT, HI and DEIA. All sera were titrated and reciprocal endpoint titers recorded. Table 1 shows that 78.7% of the sera were positive by PRNT, 66.0% were positive by HI and 79.8% were positive by DEIA. Clearly the sensitivity of the DEIA test is similar to that of the PRNT while the HI test is less sensitive than either the DEIA or PRNT.

The PRNT is considered to be more specific and more sensitive than the HI test. It was thus important to compare the DEIA with the PRNT to determine its specificity and sensitivity. Seventy-seven pig sera negative at 1 : 200 and 192 sera positive at 1 : 200 for JEV using the DEIA test

Table 1

Comparison of sensitivity of PRNT, HI and DEIA.

	Pos (%)	Neg (%)	Total
PRNT	74 (78.7)	20 (21.3)	94
HI	62 (66.0)	32 (34.0)	94
DEIA	75 (79.8)	19 (20.2)	94

were screened for neutralizing antibodies to JEV at 1 : 10 dilution. As shown in Table 2, 51 of the 77 DEIA negative sera (66.2%) did not have neutralizing antibodies to JEV, but 23 of the 26 DEIA negative sera had very low PRNT titers of 1 : 10. Of the 192 specimens which were positive for antibodies to JEV, 178 or 92.7% were also positive by PRNT. The 14 which were PRNT negative had very low DEIA titers of 1 : 200.

The positive results obtained by any of the methods used (HI, DEIA or PRNT) would only be suggestive of previous exposure to JEV, and considering that the pigs were 6 to 9 months of age when tested, these data show that there had been JEV transmission among the pigs during the 6 to 9 months prior to testing. This is not a disadvantage when sentinel animals are being screened since serial bleeds from each animal would be available and thus continuous and regular screening of the sentinels would indicate when JEV activity would be occuring.

However, when pig populations are screened at regular intervals, it is an advantage to know how much active infection has been occuring in relation to prior exposure. In order to address this question, the presence of IgM was determined in all the 175 pig sera collected during December 1989. There were 7 collections during which 25 specimens were collected each time. 12 sera were positive for IgM to JEV and these clustered in the first 8 days of December as shown in Fig 1. The reciprocal endpoint titers by DEIA and PRNT of each IgM positive serum are shown in Table 3.

Table 2

Comparison of reciprocal endpoint titers by PRNT and DEIA.

PRNT	<10	10	40	160	640	2560	total
DEIA							
<200	51	23	1	2	0	0	77
200	14	21	11	3	0	0	49
500	0	0	1	3	2	0	6
1000	0	0	12	79	25	3	119
2000	0	0	0	1	7	0	8
≥4000	0	0	0	3	7	0	10
Total	65	44	25	91	41	3	269

One pig (not shown) had a Ag/C ratio of 1.6 and was below the cutoff ratio of 2. However, the DEIA titer was 1 : 1000 while the PRNT titer was as high as 1 : 2560 indicating a recent infection with IgM levels waning concomitant with rising neutralizing antibodies.

Fig 1 also demonstrates that the percentage of pigs with high titered IgG (> = 1 : 1000) as determined by DEIA increases shortly after the IgM cluster as does the percentage of pigs with high titered neutralizing antibodies (> = 1 : 160). Furthermore, the 3 outliers in Table 2 where the DEIA titers were less than 1 : 200 with neutralizing antibody titers of 1 : 40 (1 serum) and 1 : 160 (2 sera), were found to be all clustered during the week following the IgM positive cluster. This suggests that there was intense JEV transmission dur-

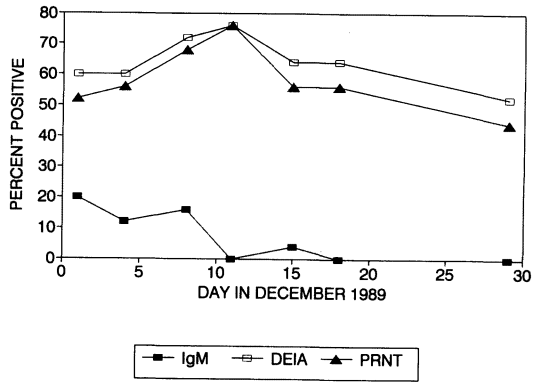

Fig 1—Antibodies to JEV in pigs, December 1989.

Table 3

DEIA and PRNT titers of IgM positive sera.

Serum no.	AG/C	DEIA	PRNT
2111	2.5	1000	640
2112	4.6	1000	640
2113	3.5	1000	160
2124	29.8	<200	10
2209	3.2	1000	160
2212	9.7	<200	<10
2221	8.7	1000	640
2318	2.5	1000	40
2319	3.2	10,000	160
2322	4.1	1000	160
2325	3.3	1000	160
2507	4.0	1000	160

ing late November and early December 1989 and that it might be possible to predict periods of intense JEV transmission by monitoring relative numbers of high titered DEIA antibodies in a population of pigs.

This study was not meant to be an epidemiological study of transmission of JEV among pigs, but the IgM data suggests that a long term year-round study should be carried out to determine the pattern of JEV transmission in Penang, Malaysia.

To address the question of specificity with respect to the other major flavivirus circulating in the region, 20 high titer sera were selected and titrated by DEIA and by PRNT using dengue virus antigens and dengue 2 virus respectively. These titers were compared with the titers obtained by using JEV. Table 4 lists these data, and it is clear that while the sera all had strong reactions with JEV antigens in the DEIA, they reacted very weakly or not at all with dengue virus antigens. In the PRNT comparison, all the sera had neutralizing titers of 1 : 640 or more (1 serum), against JEV but all except 2 did not neutralize dengue 2 virus. The two which did only had a titer of 1 : 10 against DEN2 compared to 1 : 640 and 1 : 2,560 against JEV. There is little doubt that these pigs had been exposed to JEV and that the DEIA test for JEV antibodies is very specific when used for screening for antibodies against JEV in situations where homologous infection is expected.

Table 4

Comparison of DEIA and PRNT titers using JE and dengue viruses.

Specimen no.	DEIA titer		PRNT titer	
	JE AG	DEN AG	JEV	DEN 2
1122	1 : 2000	< 1 : 200	1 : 640	< 1 : 10
1124	1 : 500	< 1 : 200	1 : 640	< 1 : 10
1209	1 : 4000	1 : 200	1 : 640	< 1 : 10
1210	1 : 2000	1 : 200	1 : 640	< 1 : 10
1224	1 : 2000	1 : 200	1 : 640	< 1 : 10
1314	1 : 1000	1 : 200	1 : 640	< 1 : 10
1316	1 : 1000	< 1 : 200	1 : 640	< 1 : 10
1403	1 : 2000	1 : 200	1 : 640	< 1 : 10
1407	1 : 1000	1 : 200	1 : 640	< 1 : 10
1411	1 : 2000	1 : 200	1 : 640	< 1 : 10
1424	1 : 2000	1 : 200	1 : 640	< 1 : 10
1513	1 : 500	< 1 : 200	1 : 640	< 1 : 10
1514	1 : 1000	1 : 200	1 : 640	< 1 : 10
1718	1 : 4000	1 : 200	1 : 640	< 1 : 10
1801	1 : 2000	< 1 : 200	1 : 640	< 1 : 10
2407	1 : 1000	< 1 : 200	1 : 640	1 : 10
2507	1 : 1000	< 1 : 200	1 : 640	< 1 : 10
2508	1 : 1000	1 : 200	1 : 640	< 1 : 10
2509	1 : 1000	1 : 500	1 : 640	< 1 : 10
2609	1 : 1000	1 : 500	1 : 2560	1 : 10

In conclusion, the DEIA may be usefully employed for screening animal sera for the presence of antibodies to JEV. It compares well to the PRNT method, and in fact is more sensitive than the HI test when both these tests are compared with the PRNT. However, the main advantage of using the DEIA test is that it is simple to perform, does not require cell culture facilities at the point of testing as does the PRNT, nor does it require the use of fresh goose erythrocytes, labile antigens and pH optimisation as does the HI test. Further, the antigen coated membranes are very stable, and in our experience may be stored for at least two years at 4°C without deterioration. The test may also be carried out with minimal training and experience and is highly portable, making it useful as a field surveillance tool.

When used in conjunction with IgM screening, more detailed information about transmission can be obtained and may prove useful in planning control measures.

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