DEVELOPMENT OF AN ELISA FOR THE DETECTION OF ANTIBODIES TO JEMBRANA DISEASE IN BALI CATTLE

NINING HARTANINGSIH, G. E. WILCOX, M. TENAYA and S. SOEHARSONO
Bali Cattle Disease Investigation Unit - IFAD, Denpasar, Bali, Indonesia

ABSTRACT

Hartaningsih, N., G. E. Wilcox, M. Tenaya and S. Soeharsono. 1993. Development of an ELISA for the detection of antibodies to Jembrana disease in Bali cattle. *Penyakit Hewan* 25 (46A): 37-43.

An enzyme-linked immunosorbent assay (ELISA) has been developed for the detection of antibodies specific for the Jembrana disease agent. Virus present in plasma of experimentally infected cattle and purified by sucrose density gradient centrifugation was used as antigen. In experimentally infected Bali cattle, antibody was detected as early as 8 weeks after infection and the majority of cattle were antibody positive 3 months after infection. The antibody persisted for more than 59 weeks. A serological survey was conducted of cattle from various areas in Indonesia to determine the distribution and prevalence of cattle with antibodies to Jembrana disease virus. Antibodies were detected in serum samples from cattle from five provinces, Bali, Lampung, East Java (Banyuwangi), West Sumatera and South Kalimantan. This finding correlated with the reported occurrence of clinical Jembrana disease.

Key words: ELISA, antibodi, Jembrana disease, Bali cattle

ABSTRAK

Hartaningsih, N., G. E. Wilcox, M. Tenaya dan S. Soeharsono. 1993. Pengembangan ELISA untuk deteksi antibodi terhadap penyakit Jembrana pada sapi Bali. *Penyakit Hewan* 25 (46A): 37-43.

Suatu ELISA telah dikembangkan untuk mendeteksi antibodi yang spesifik dari agen penyakit Jembrana. Virus yang berada dalam plasma dari sapi yang terinfeksi secara eksperimental dan telah dimurnikan dengan menggunakan sentrifugasi *sucrose denisity gradient* digunakan sebagai antigen. Pada sapi Bali yang diinfeksi secara ekspermental, antibodi dapat dideteksi pada 8 minggu setelah infeksi dan sebagian besar sapi mempunyai antibodi positip pada waktu 3 bulan setelah infeksi. Antibodi bertahan untuk lebih dari 59 minggu. Suatu survei serologis telah dilakukan pada sapi dari berbagai daerah di Indonesia untuk menentukan distribusi dan prevalensi sapi yang mempunyai antibodi terhadap virus penyakit Jembrana. Antibodi dapat dideteksi dalam contoh serum sapi asal 5 provinsi yaitu Bali, Lampung, Jawa Timur (Banyuwangi), Sumatera Barat dan Kalimantan Selatan. Hasil temuan ini berkaitan dengan laporan kejadian penyakit Jembrana secara klinis.

Kata kunci: ELISA, antibodi, penyakit Jembrana, sapi Bali

INTRODUCTION

Jembrana disease is a disease naturally affecting mainly Bali cattle (*Bos zondaicus*). The clinical and pathological pictures are characterised by fever, leucopoenia, diarrhoea, haemorrhages in various tissues, lymphadenopathy and splenomegaly (Soeharsono *et al.*, 1990; Soesanto *et al.*, 1990; Dharma *et al.*, 1991).

The aetiological agent of Jembrana disease has been identified as a retrovirus based on the morphology in electron microscopy, the presence of reverse transcriptase activity and the detection of a specific protein band by a western blot (Wilcox *et al.*, 1992). There is evidence of antigenic relationship between the Jembrana disease virus and bovine immunodeficiency virus (BIV) (Brownlie and Wilcox pers. comm.).

Field diagnosis of Jembrana disease is usually based upon epidemiology, clinical signs and gross pathology. Laboratory confirmation is mainly based on histopathology. This paper describes the development of an ELISA for the detection of antibody against the Jembrana disease virus.

MATERIALS AND METHODS

Preparation of antigen

Susceptible cattle were inoculated intravenously with Jembrana disease virus prepared as described by Soeharsono *et al.* (1990). Heparinised blood was obtained from experimentally infected cattle on the second day of the febrile reaction, approximately 7 days post-inoculation. The plasma was separated by centrifugation at 2100 *g* for 15 min. in a Beckman L7-70 ultracentrifuge (type 35 rotor) (Beckman Instruments Inc., Palo Alto, USA) and the supernatant centrifuged again at 96,000 *g* for 2 hours in a Beckmann L7-70 ultracentrifuge (SW 28

rotor). The pellet was resuspended in a solution of 10 mM Tris/50 mM NaCl pH 7.2 (TBS) and centrifuged again at 96,000 *g* for 2 hours. The final pellet was resuspended in TBS to 0.5% of the original volume. Plasma from normal cattle was processed in a similar manner. Some of these pellets were frozen at -20°C and used as a crude antigen in blocking and competition ELISAs.

Additional centrifuged pellets of plasma of normal and infected cattle, prepared as described above, were layered on a continuous 10% to 60% (w/v) sucrose gradient using TBS as a diluent and centrifuged at 96,000 *g* for 4 hours in a Beckman L7-70 centrifuge (SW 28 rotor). The band present at a density of approximately 1.15 g mL^{-1} in plasma from infected cattle was removed from the sucrose gradient, dialysed against TBS overnight, concentrated to 1 % of the original volume by reverse dialysis against polyethylene glycol, dispensed in aliquots and stored frozen at -20°C. When required, aliquots were thawed and used as the antigen for ELISA.

Serum

Two control positive sera were prepared. One positive serum (577) was prepared by hyperimmunising a Bali animal with 10 subcutaneous injections at weekly intervals of pelleted plasma antigen in TBS. Prior to injection, the plasma was treated with 1 % formaldehyde for 24 hours at 37°C to inactivate the virus and homogenised with an equal volume of incomplete Freund's adjuvant. Serum was collected 7 days after the last injection. A second reference positive serum (Pan Moyo) was obtained from a naturally infected animal in the endemic Jembrana disease area of Tabanan, Bali. The sera were diluted 1:10 in 0.1 M phosphate buffered saline with 0.05 % Tween 20 pH 7.2 (PBST), dispensed in aliquots and stored frozen at -20°C.

Pre-infection, negative control sera were obtained from 100 Bali cattle purchased from Nusa Penida island, where Jembrana disease is not known to occur. Immune sera were collected from 20 cattle at various intervals after they had recovered from experimentally induced Jembrana disease.

Nineteen additional Bali cattle were experimentally infected with the Tabanan/87 strain of Jembrana disease virus. All of these cattle developed clinical signs typical of Jembrana disease. Sera were collected from all cattle 3 days before infection and then at 2 week intervals until 59 weeks after infection. All sera were stored at -20°C prior to use.

ELISA

An optimal concentration of sucrose gradient purified viral antigen in carbonate-bicarbonate buffer pH 9.6 was added to each well of 96-well polystyrene flat bottom microtitre plates (Linbro EIA plates, Flow Laboratories, Inglewood, CA, U.S.A.) in 100 µL amounts and incubated at 4°C overnight. The coated plates were washed 4 times at 15 second intervals with PBST using an automatic plate washer (Flow Laboratories, U.S.A). Excess washing solution was removed, and 100 µL of each test serum was then added to the appropriate wells. On each plate, appropriate dilutions of positive (Pan Moyo) and negative reference serum and a diluent control were included. The plates were incubated at 37°C for 1 hour, then washed 4 times with PBST and 50 µL of an optimal concentration (1:500) of alkaline phosphatase conjugated monoclonal anti-bovine IgG (Silenus, Australia) diluted in PBST was added to each well. After incubation for 1 hour at 37°C the plates were washed 4 times with PBST and 100 µL of p-nitrophenyl phosphate in diethanolamine buffer substrate solution was added. The plates were incubated at 37°C for 1 hour after which the optical density (OD) of the reaction in each well was read with a Titertek Multiskan (Flow Laboratories, U.S.A.) at a wavelength of 405 nm. The reactions were blanked with the diluent controls.

The optimal dilution of antigen to be used in the ELISA was determined by titrating two-fold dilutions, 1:20 to 1:640, against a 1:20 dilution of #577 reference positive sera. The optimal dilution of antigen was then tested against two-fold dilutions of #577 serum, the Pan Moyo reference positive antiserum and a serum pool from susceptible cattle prior to infection (Nusa Penida).

The diagnostic cut-off was taken as the mean plus 3 standard deviations (SD) of the OD of the Pan Moyo reference positive serum at a dilution of 1:640. This base OD was the same as the mean OD for a 1:20 dilution of the negative sera from the 100 cattle susceptible to infection.

ELISA results were quantitated by expressing them as the ratio - (mean OD of the test serum/mean OD + 3SD of a 1:640 dilution of the Pan Moyo reference positive serum). The specificity of the ELISA reaction with the 2 reference sera was tested by a competition and a blocking ELISA.

Competition ELISA

Fifty microlitres of an optimal dilution of either a positive or negative crude positive antigen or PBST and

50 µL of 1:10 dilutions of the reference positive sera were added concurrently to wells coated with the sucrose gradient purified antigen. The ELISA was then performed as described above.

Blocking ELISA

Equal volumes of a 1:10 dilution of the reference positive sera and an optimal dilution of either a positive or negative crude antigen or PBST were mixed and incubated at 37°C for 20 hours and then 10 µL of the mixtures were added to wells coated with the sucrose gradient purified antigen. The ELISA was then performed as described above. The ratio of the OD of the reaction of sera mixed PBST and the sera mixed with the crude antigens was determined. The optimal dilution of the crude antigens used in the assays was determined by titration as the maximum dilution of the positive antigen which caused an 80% or greater reduction in the OD of the reference Pan Moyo serum.

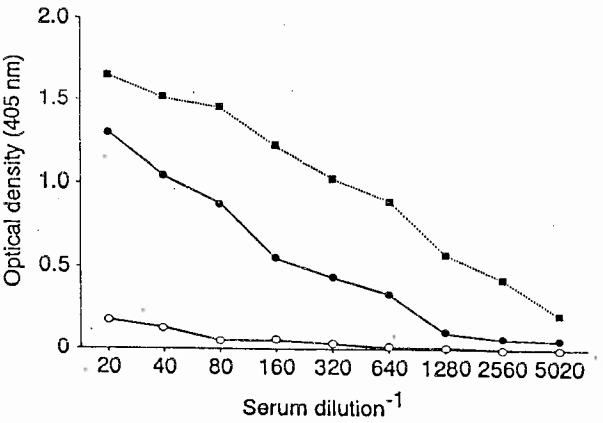

Figure 1. Titration of sample sera against an optimal dilution of Jembrana disease's antigen. • Nusa Penida (negative control pool, n=6); • Pan Moyo (positive field control); ■ 577 (hyperimmunised control)

RESULTS

Cattle previously experimentally infected with Jembrana disease were rechallenged with Jembrana disease virus on the day of serum collection to determine their susceptibility to Jembrana disease. Only one of the 20 cattle developed clinical disease, indicating that the animals were resistant to infection.

The validity of using the mean + 3SD OD value as a diagnostic cut-off was confirmed by retesting the pre-infection, negative control serum samples obtained from 100 animals from a known Jembrana disease-free area. All of these animals were subsequently challenged with the Jembrana disease virus and all developed the disease (data not presented).

Using the indirect ELISA, the two reference positive sera (577 and Pan Moyo) reacted with the sucrose gradient purified viral antigen whereas the negative sera produced only minimal background reactions (Figure 1). A 1:640 dilution of the reference positive Pan Moyo serum gave an OD reading approximately the same mean OD of 1:20 dilution of the 100 reference negative sera.

The mean + 3SD OD of a 1:640 dilution of the positive reference Pan Moyo serum was chosen as the minimum OD value of a positive reaction in the ELISA. When a serum dilution of 1:20 was used for the detection of antibodies to Jembrana disease virus, all 100 samples from cattle susceptible to Jembrana disease gave an OD reading less than this value.

In the 20 sera from animals previously infected with Jembrana disease virus, 17 sera from animals which were resistant to challenge with Jembrana disease virus had an OD value greater than the cut-off OD and 3 of the 20 sera gave an OD value less than the cut-off. Two of the sera were from animals which were also resistant to challenge with the Jembrana disease and 1 was from an animal (CB 641) which remained susceptible to challenge with Jembrana disease virus (Table 1).

The results of the competition and blocking ELISA with the 2 reference positive sera are shown in Table 2. The addition of a 1:32 dilution of a crude antigen prepared by the centrifugation of plasma from infected cattle reduced the OD of the Pan Moyo reference serum by 80 % and 91 % in the blocking and competition ELISAs, respectively. The OD of the Pan Moyo reference serum was reduced by 21 % in the competition ELISA only when a negative antigen was used.

The OD of the reference serum prepared by hyperimmunisation of an animal (577) with a crude antigen was reduced after addition of the crude positive antigen by 50% and 38% in the blocking and competition ELISAs, respectively. Inhibition was also observed with both the blocking (9%) and competition (21%) ELISAs when a negative antigen preparation was tested with this serum. The addition of larger amounts of both the negative and positive crude antigens to #577 resulted in a greater reduction in the OD values with both antigens, but the ratios of the differences between both antigens remained similar (results not shown).

Table 1. Relationship of the ELISA result to the immune status of Bali cattle challenged with Jembrana disease virus

Animal Identification	ELISA results		immune status of animal[1]
	OD (405 nm)	OD Ratio[2]	
CB407	1.008	7.2	Resistant
CB485	0.913	6.5	Resistant
CB484	0.800	5.7	Resistant
CB186	0.783	5.6	Resistant
CB478	0.761	5.4	Resistant
CB460	0.749	5.3	Resistant
CB502	0.739	5.3	Resistant
CB442	0.722	5.2	Resistant
CB512	0.679	4.8	Resistant
CB510	0.670	4.8	Resistant
CB518	0.644	4.6	Resistant
CB492	0.609	4.3	Resistant
CB523	0.573	4.1	Resistant
CB514	0.437	3.1	Resistant
CB525	0.306	2.2	Resistant
CB521	0.174	1.2	Resistant
CB508	0.151	0.1	Resistant
CB461	0.111	0.7	Susceptible
CB524	0.098	0.7	Resistant
CB451	0.076	0.5	Resistant

[1] Determined by challenge of the recovered cattle with Jembrana disease virus

[2] Ratio of the OD of the test serum to the OD of the reference positive (Pan Moyo) serum at a dilution of 1:640

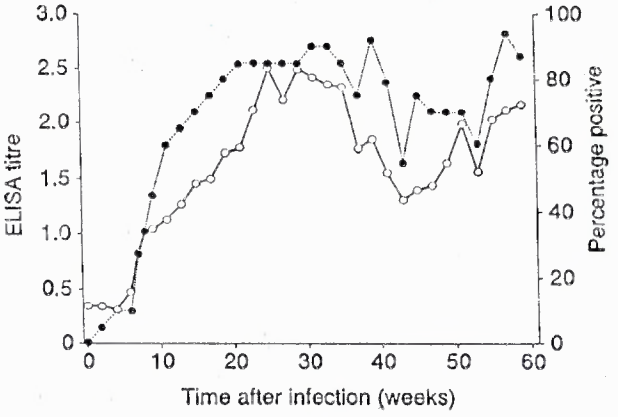

Figure 2. Time course of antibody response to Jembrana disease virus in Bali cattle. ELISA titres are expressed as the ratio (OD of the test sera/OD of a 1:640 dilution of the reference positive Pan Moyo serum); a value of 1.0 or greater indicates a positive result. • ELISA titre; • percentage positive (n=20)

Immune response in experimentally infected Bali cattle

The ELISA results using sera from Bali cattle which were experimentally infected and developed Jembrana disease are shown in Figure 2. Antibody was not detected in any cattle at the time of challenge. Antibody was detected in 18 of the 19 cattle after infection; antibody was never detected in the remaining animal.

A positive ELISA result was initially detected in a low percentage of the cattle 2 weeks after challenge but it was not until 8 weeks after challenge that the mean OD of the sera from all animals exceeded that of the positive/negative discrimination value and it was only after 11 weeks post-challenge that sera from a majority of the cattle gave a positive result. The maximum number of positive cattle and the peak mean OD of the sera occurred between 23 and 33 weeks post-challenge. Antibody persisted until 59 weeks post-challenge, when the observations were discontinued, in a majority of the cattle. There was a transient decrease between 29 and 55 weeks post-challenge in the number ofantibody-positive cattle and the mean titre of antibody detected by the ELISA.

Table 2. Specificity of the Jembrana disease blocking and competition ELISAs

ELISA type	Reference Serum	PBST OD	ELISA result[1]			
			Negative antigen		Positive antigen	
			OD	% inhibition	OD	% inhibition
Blocking	Pan Moyo[2]	1.18	1.18	0	0.24	80
	#577[3]	1.61	1.45	9	0.81	50
Competition	Pan Moyo	1.08	0.86	21	0.11	91
	#577	1.39	1.10	21	0.88	38

[1] In the presence of PBST, or 1:32 dilutions of crude positive or negative antigen

[2] Naturally exposed animal

[3] Hyperimmunised animal

Table 3. Prevalence of Jembrana disease ELISA antibodies in cattle in Indonesia

Province	District	Sub-district	Cattle breed					
			Bali		Ongole		Bali X[1]	
Lampung	Lampung Selatan	Natar	0[2]	(514)[3]	-		-	
		Palas	19	(218)	-		-	
		Sidomulyo	5	(294)	-		-	
	Lampung Tengah	Punggur	6	(17)	-		-	
		Seputih Raman	29	(371)	0	(2)	33	(3)
		Seputih Mataram	13	(146)	12	(25)	0	(25)
		Seputih Banyak	17	(441)	7	(15)	-	
		Raman Utara	15	(180)	33	(3)	0	(1)
		Rumbia	4	(521)	-		-	
		Seputih Surabaya	6	(332)	-		-	
		Sekampung	33	(49)	-		-	
		Labuan Maringgai	0	(60)	-		-	
Sumatera		Sawahlunto/Sijunjung	14	(201)	-		-	
East Java	Madura Island/Banyuwangi.		31	(100)	-	0		(301)
Kalimantan	Kalimantan SouthBanjarbaru		33	(24)	-		-	
Jambi			0	(208)	-		-	
South Sulawesi			0	(201)	-		-	
Nusa Tengah Timor			0	(214)	-		-	
Nusa Tengah Barat			0	(155)	-		-	

[1] Bali/Ongole cross
[2] Percentage ELISA positive
[3] No. tested

Table 4. Comparison of Jembrana disease ELISA results and reported clinical cases of disease in Bali

District	Cattle population	Prevalence of Jembrana disease				Deaths
		By ELISA		Field outbreaks		
		%[1]	(n)[2]	Cases	%[3]	
Jembrana	26,831	27	(150)	170	(0.63)	9
Tabanan	44,287	22	(149)	281	(0.63)	41
Buleleng	82,508	17	(150)	562	(0.68)	32
Karangasem	93,457	15	(150)	128	(0.13)	26
Gianyar	53,453	14	(28)	31	(0.06)	1
Bangli	69,780	13	(150)	26	(0.04)	1
Klungkung	36,916	6	(137)	0	(0)	0
Badung	48,947	3	(153)	0	(0)	2
Nusa Penida[4]	-	0	(100)	0	(0)	0
Total	456,179[5]	117	1167	1198[5]	(0.26)	112

[1] Percentage of samples ELISA positive
[2] Number of samples tested
[3] Percentage of cattle population
[4] Nusa Penida Island is classified as a sub-district of Klungkung
[5] Not included in calculations

Serological survey

The serological survey indicated that antibodies were detected in serum samples from cattle in 5 provinces of Indonesia: Bali, East Java, Lampung, West Sumatera and South Kalimantan (Table 3). In Lampung, antibodies were detected only in the Lampung Tengah and Lampung Tengah (especially Seputih Raman, Seputih Banyak and Sekampung Sub-districts) Districts but not in the adjacent District of Lampung Utara. Antibody-positive animals were also detected recently in West Sumatera and South Kalimantan. In East Java, antibodies were detected incattle in the Banyuwangi district but not in cattle from the island of Madura. In Bali province, antibodies were detected in all districts on the island but not on the adjacent island of Nusa Penida. There was a higher prevalence of antibody-positive cattle in western districts (Jembrana, Tabanan and Bulcleng) than other districts, which corresponds to the reported prevalence of clinical cases of Jembrana disease in cattle in Bali (Table 4).

DISCUSSION

While Jembrana disease virus has been demonstrated to replicate in non-adherent mononuclear cells of peripheral blood origin it has not been possible to obtain sufficient virus from cell culture for the preparation of an antigen for serological tests. The use of plasma from infected cattle as a source of virus for antigen provided large volumes of material with a high titre of virus. Partial purification of this material was achieved by sucrose density gradient centrifugation of the plasma and the use of the visible band at a density of 1.15 g/ml. A high concentration of viral particles was present in this band (data not presented).

The ELISA was specific as it detected antibody only in cattle which had been previously infected with Jembrana disease. Antibody was not detected in cattle from Nusa Penida, an island adjacent to Bali, where Jembrana disease has not been reported to occur, and whose animals are consistently susceptible to challenge with Jembrana disease virus. However, after infection, these cattle developed antibody detectable by the ELISA.

Additional evidence of the specificity was provided by the observation that the ELISA reaction of positive sera was blocked by the addition of a crude virus-containing plasma preparation, but not by a similar antigen prepared from uninfected cattle. The specificity of the blocking test was greater with the reference serum from a naturally infected animal than with the reference serum produced by hyperimmunisation of an animal with a crude virus preparation, presumably due to the presence of antibodies against bovine proteins in the latter.

Although antibody was detected in a low percentage of experimentally infected Bali cattle in the 6 weeks post-challenge, antibody was not detected in the majority of the infected cattle until 11 weeks post-challenge and a maximal response was not detected until 23 to 33 weeks post-challenge. The antibody then persisted in a majority of the cattle until 59 weeks post-challenge, when the sequential study was terminated. The long period post-challenge before an antibody response was detected in a majority of the cattle by the ELISA may be partly caused by the use of a monoclonal anti-IgG conjugate in the ELISA. Although IgM may be produced in the period before IgG production, attempts to detect Jembrana disease virus-specific IgM in recovered cattle using similar ELISA have been unsuccessful (data not presented).

Jembrana disease is an acute disease with clinical signs occurring 4-12 days after infection and persisting for 5 to 12 days (Soesanto, *et al.*, 1990; Soeharsono, *et al.*, 1990). More than 80 % of cattle survive the infection and are clinically normal thereafter and resistant to reinfection (Soeharsono *et al.*, 1990). There is a persistent low level viraemia in the recovered cattle. The immunological mechanism involved in recovery from the disease is unknown, but the inability to detect antibody in the cattle until 6 weeks after infection (3-4 weeks after recovery from the disease) suggests that the mechanism of recovery from the acute disease is not antibody-mediated.

As "Ramadewa" disease and "Banyuwangi" disease in Lampung province and East Java, respectively, are clinically and pathologically indistinguishable from Jembrana disease, the detection of antibodies against Jembrana disease virus in the areas where these diseases occur, and not in other areas of Indonesia, suggests that all of these diseases are closely related or the same.

In Bali, the reported prevalence of Jembrana disease cases is higher in the western district (Jembrana, Tabanan and Buleleng) then in other districts of Bali island. The reason for this is unknown, although there was a correlation between the prevalence of cattle in Bali with antibody to Jembrana disease and the number of reported cases of Jembrana disease (Table 4). There have been no reported cases of Jembrana disease and no serological evidence was obtained of the disease in the Nusa Penida district of Bali province.

In Lampung Tengah, there was also a correlation between the prevalence of serologically positive cattle and the presence of the disease. There was a higher prevalence of serologically positive animals in the Seputih Raman, Seputih Banyak and Sekampung sub-districts; the majority of clinical cases of "Ramadewa" disease are reported to occur in Seputih Raman and Seputih Banyak and an outbreak of the disease has recently occurred in the Palas and Sidomulyo Sub-districts of Lampung Selatan. New outbreaks of Jembrana disease, as diagnosed clinically, histopathologically and serologically, occurred in April 1992 in Sawahlunto/ Sijunjung, Sumatera Barat (Anonymous, 1992). A similar disease which was diagnosed only on clinical grounds occurred in Kalimantan Selatan in 1991. There have been no reports of clinical Jembrana disease or other disease indistinguishable from Jembrana disease in cattle other than Bali cattle. However, in the current study there was serological evidence that Ongole (*Bos indicus*) and cross-bred Bali cattle in Lampung Tengah have been exposed to Jembrana disease virus. Although clinical disease in Ongole cattle has not been reported, the presence of antibodies in these cattle is consistent with the report that Ongole cattle, when experimentally infected with Jembrana disease virus, develop a subclinical disease and viraemia which persists for up to 6 months after infection (Socharsono *et al.*, 1990).

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