ORIGINAL ARTICLE

Detection of liver kidney microsomal type 1 antibody using molecularly based immunoassays

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Aims: To assess the diagnostic value of two commercial molecularly based immunoassays detecting liver kidney microsomal type 1 antibody (LKM1).

Methods: The performance of Varelisa and LKM1 enzyme linked immunosorbent assay (ELISA) was compared with immunofluorescence, and two validated research techniques—an in house ELISA and a radioligand assay measuring antibodies to P4502D6. Thirty serum samples from three patients with autoimmune hepatitis type 2 covering immunofluorescence titres of 1/10 to 1/10 240 and 55 LKM1 negative controls were tested.

Results: All 30 sera that were LKM1 positive by immunofluorescence were positive by the in house ELISA, the radioligand assay, and LKM1-ELISA, and 29 were also positive by Varelisa. None of the 55 sera negative for LKM1 by immunofluorescence was positive by the in house ELISA and radioligand assay, but one was positive by Varelisa and 14 were positive using the LKM1-ELISA. Agreement between immunofluorescence, the in house ELISA, the radioligand assay, and Varelisa was high ($\kappa > 0.8$), and agreement between immunofluorescence and LKM1-ELISA was moderate ($\kappa = 0.63$).

Conclusion: The assay kit marketed as Varelisa allows accurate detection of LKM1.

Liver kidney microsomal antibody type 1 (LKM1) is the diagnostic marker of a severe form of autoimmune hepatitis—autoimmune hepatitis type 2 (AIH2)—which typically affects children and young adults.[1] LKM1 is conventionally detected by immunofluorescence (IFL), a subjective technique, using rat liver, kidney, and stomach as a composite substrate.[2] Because of its rarity and staining similarity with antimitochondrial antibody and liver cytosol type 1 antibody, LKM1 is often misidentified.[3–6] Since the identification of the target of LKM1 as cytochrome P4502D6 (CYP2D6),[7–9] an enzyme responsible for the metabolism of several drugs, instrumentally based, objective assays have been established. However, these assays, which use eukaryotically expressed CYP2D6, have been tailored to the requirements of research establishments. Thus, radioligand assays (RLAs),[10 11] considered to be the gold standard for the detection of LKM1, are complex and labourious, and an enzyme linked immunosorbent assay (ELISA)[12] established in our laboratory, although both sensitive and specific, requires repeated standardisation with each batch of antigen/reagents and is not commercially available. The aim of our present study was to assess the potential diagnostic value of two commercial assays detecting LKM1: Varelisa (Pharmacia and Upjohn Diagnostics, Freiburg, Germany), which uses baculovirus/insect expressed CYP2D6, and LKM1-ELISA (Medical and Biological Laboratories, Nagoya, Japan), which uses prokaryotically expressed CYP2D6. The proficiency of these two assays has not been independently tested to date. Thirty sera, taken at different stages of disease activity from three children with AIH2, and ranging in LKM1 titre from 1/10 240 to 1/10 (as assessed by immunofluorescence), were investigated with these commercial kits and the results were compared with those obtained with standard immunofluorescence, in house ELISA, and RLA.

> "Radioligand assays, considered to be the gold standard for the detection of LKM1, are complex and labourious"

MATERIALS AND METHODS

Thirty serum samples from three female patients with LKM1 positive AIH were selected on the basis that on routine immunofluorescence testing[13] they were found to cover the LKM1 titre range of 1/10 to 1/10 240 (table 1). An aliquot of these samples, stored at −70°C, was tested using the four assays: in house ELISA, RLA, and the two commercial ELISAs. The three patients from whom the 30 sera were obtained had classic AIH2, diagnosed according to the criteria of the international autoimmune hepatitis group.[14] Two sera were taken at the time of diagnosis, six during relapse, and the remaining 22 while in remission. Two patients had other autoimmune disorders: insulin dependent diabetes mellitus in one and ulcerative colitis in the other. Sera from 45 LKM1 negative patients were also tested as pathological controls. Twenty nine had other autoimmune liver diseases (age range, 4.3–18.6 years; median, 13.5; 14 females), 15 having antinuclear antibody (ANA) and/or antismooth muscle antibody (SMA) positive AIH (autoimmune hepatitis type 1; AIH1), and 14 ANA/SMA positive sclerosing cholangitis with characteristic bile duct changes on cholangiography. One girl with AIH1, who was persistently positive for SMA at diagnosis and follow up, was positive on a single occasion five years before our present study for LKM1 by IFL at a titre of 1/10 and for anti-CYP2D6 by RLA. Sixteen patients with non-autoimmune liver disease were also tested, eight having Alagille syndrome (age range, 2–9.6 years; median, 5.8; four females) and eight α1 antitrypsin deficiency (α1ATD; age range, 10–14 years; median, 6.4; three females). As normal controls, sera from 10 healthy children (age range, 4.3–18.6 years; median, 11.9; five females) were studied.

ELISA

The antigen used in the in house ELISA[12] is eukaryotically expressed solid phase CYP2D6. A cutoff point of 0.16 arbitrary

Abbreviations: AIH1, autoimmune hepatitis type 1; AIH2, autoimmune hepatitis type 2; ANA, antinuclear antibody; AU, arbitrary units; cpm, counts per minute; CYP2D6, cytochrome P4502D6; ELISA, enzyme linked immunosorbent assay; IFL, immunofluorescence; LKM1, liver kidney microsomal antibody type 1; RLA, radioligand assay; SMA, antismooth muscle antibody

Table 1 Raw data obtained when the sera were tested by immunofluorescence (IFL), in house enzyme linked immunosorbent assay (ELISA), Varelisa, LKM1-ELISA, and radioligand assay (RLA)

Diagnosis	IFL LKM1 (titre)	In house ELISA (AU/ml; NV <0.16)	Varelisa (AU/ml; NV <0.55)	LKM1- ELISA (AU/ml; NV <10.78)	RLA (cpm; NV <718)
AIH2	**1/80**	**0.37**	**0.78**	**100**	**1430**
	1/160	**0.44**	**0.82**	**108.8**	**1566**
	1/640	**0.45**	**1.43**	**125**	**1623**
	1/160	**0.45**	**1.1**	**108.8**	**1331**
	1/320	**0.47**	**1.1**	**112.5**	**1515**
	1/2560	**0.61**	**1.44**	**137.5**	**1403**
	1/2560	**0.66**	**1.42**	**135**	**1649**
	1/10 240	**0.65**	**1.45**	**137.5**	**1795**
	1/10 240	**0.65**	**1.45**	**125**	**1775**
	1/2560	**0.66**	**1.43**	**137.5**	**1775**
	1/2560	**0.64**	**1.42**	**137.5**	**1841**
	1/1280	**0.64**	**1.44**	**137.5**	**1738**
	1/160	**0.6**	**1.41**	**137.5**	**1663**
	1/640	**0.51**	**1.24**	**125**	**1804**
	1/40	**0.51**	**1.13**	**125**	**1788**
	1/320	**0.53**	**0.79**	**100**	**1810**
	1/40	**0.51**	**0.7**	**100**	**1830**
	1/80	**0.42**	**0.68**	**100**	**1572**
	1/80	**0.37**	**0.59**	**95**	**1590**
	1/80	**0.36**	0.5	**93**	**1776**
	1/1280	**0.35**	**1.43**	**125**	**1834**
	1/40	**0.45**	**0.79**	**83.7**	**1797**
	1/40	**0.36**	**0.72**	**75**	**1526**
	1/10	**0.4**	**0.64**	**92.5**	**1288**
	1/10	**0.44**	**0.64**	**95**	**1465**
	1/80	**0.47**	**1.15**	**121.2**	**1677**
	1/640	**0.4**	**1.33**	**115**	**1562**
	1/80	**0.47**	**1.01**	**112.5**	**1623**
	1/640	**0.47**	**1.39**	**117.5**	**1808**
	1/640	**0.48**	**1.44**	**132.5**	**1343**
AIH1	Neg	0.01	0.07	**12.5**	634
	Neg	0.04	0.08	*7.5*	*706*
	Neg	0.06	0.05	3.8	687
	Neg	0.02	0.05	10	643
	Neg	0.05	0.06	8.8	590
	Neg	0.03	0.05	8.8	441
	Neg	0.01	0.1	**16.2**	473
	Neg	0.02	0.12	**12.5**	527
	Neg	0.02	0.07	**17.5**	572
	Neg	0.04	0.06	**16.3**	377
	Neg	0.05	0.05	**13.5**	307
	Neg	0.03	0.03	5	218
	Neg	0.01	0.11	**15**	257
	Neg	0.08	0.08	5	323
	Neg	0.12	0.08	***35***	189
	Neg	0.12	0.14	7.5	333
	Neg	0.01	0.09	8.7	399
	Neg	0.04	**0.61**	**12.5**	241
	Neg	0.07	0.08	**12.5**	225
	Neg	0.08	0.03	6.2	200
	Neg	0.05	0.08	8.7	218
	Neg	0.09	0.07	6.2	255
	Neg	0.01	0.06	*7.5*	*275*
	Neg	0.1	0.05	6.2	243
	Neg	0.15	0.05	*7.5*	*264*
	Neg	0.04	0.05	6.3	220
	Neg	0.11	0.04	***26.2***	226
	Neg	0.03	0.05	3.7	255
	Neg	0.01	0.16	10	271
Normal	Neg	0.01	0.05	10	145
	Neg	0.06	0.05	*5*	*276*
	Neg	0.03	0.03	*5*	*229*
	Neg	0.01	0.03	2.5	200
	Neg	0.02	0.03	8.7	377
	Neg	0.02	0.05	2.5	232
	Neg	0.07	0.04	*5*	*261*
	Neg	0.01	0.03	1.3	257
	Neg	0.02	0.05	10	224
	Neg	0.02	0.04	0.5	297
α1ATD	Neg	0.04	0.04	0.5	242
	Neg	0.01	0.04	*7.5*	*244*
	Neg	0.07	0.15	**13.7**	135
	Neg	0.06	0.04	*5*	*263*
	Neg	0.03	0.05	8.7	236
	Neg	0.03	0.11	2.5	232
	Neg	0.06	0.09	10	250
	Neg	0.05	0.05	6.2	254
Alagille	Neg	0.01	0.05	**12.5**	286
	Neg	0.04	0.04	3.7	246
	Neg	0.01	0.05	2.5	237
	Neg	0.03	0.05	10	220
	Neg	0.07	0.1	**13.5**	280
	Neg	0.07	0.18	*7.5*	*275*
	Neg	0.09	0.03	*5*	*261*
	Neg	0.01	0.06	6.2	254

Cut off points were: absorbance of 0.16 AU/ml for the in house ELISA, of 0.55 AU/ml for the Varelisa, of 10.78 AU/ml for the LKM1-ELISA, and 718 cpm for the RLA. The numbers in bold denote positive results. The numbers in italic indicate positives for the LKM1-ELISA assay when the cutoff point of 18.74 AU/ml (maximum value seen with a normal control) was used.
α1ATD, α1 antitrypsin deficiency; AIH1, autoimmune hepatitis type 1; AIH2, autoimmune hepatitis type 2; Alagille, Alagille syndrome; cpm, counts per minute; NV, normal value.

Table 2 Results of the κ reliability test comparing immunofluorescence (IFL), enzyme linked immunosorbent asssay (ELISA), Varelisa, LKM1-ELISA, and radioligand assay (RLA) readings

	IFL	ELISA	Varelisa	LKM1- ELISA	RLA
IFL	1.0				
ELISA	0.98	1.0			
Varelisa	0.95	0.92	1.0		
LKM1-ELISA	0.67	0.65	0.68	1.0	
RLA	0.98	1.0	0.92	0.67	1.0

A κ value of 0.8 to 1.0 denotes very good agreement, 0.6 to 0.8 substantial agreement, and 0.4 to 0.6 moderate agreement.

units/ml (AU/ml) was used, representing the mean of values obtained from 111 controls + 3 SD × a correction factor. The correction factor was calculated by dividing the absorbance value obtained with a reference serum by the absorbance value established in previous experiments using the same reference serum.

For the commercial assays, the manufacturers' instructions were followed. The antigen used in the Varelisa kit is recombinant CYP2D6 expressed through a baculovirus/insect cell system. The cutoff point of the assay is 0.55 AU/ml, calculated as the product of the absorbance value of the positive control multiplied by a correction factor. The correction factor is obtained by dividing the expected by the observed absorbance value of the positive control provided with the assay.

The antigen used in the LKM1-ELISA kit is prokaryotically expressed, truncated CYP2D6. The cDNA encoding $CYP2D6_{125–458}$ is integrated into plasmid vector pGEX-4T and expressed in *Escherichia coli*, for the production of a glutathione S-transferase fusion protein. The antigen coated on to the plate is the fusion protein. The cutoff value is 10.78 AU/ml, representing the mean + 3 SD of the values obtained in 383 healthy controls. The manufacturer provides no correction factor. The highest value obtained by the manufacturer in a normal subject is 18.74 AU/ml.

Radioligand assay

The in house radioligand assay used in our study was based on a previously described assay.[10] Briefly, a CYP2D6 cDNA encoding full length protein (a gift from Professor U Meyer, Basel, Switzerland) was subcloned into a transcription vector pSP72. Recombinant CYP2D6 protein was then expressed in a eukaryotic in vitro transcription/translation system, metabolically labelled by ^{35}S methionine, and used in an immunoprecipitation assay. Antibodies that bound radiolabelled CYP2D6 were immunoprecipitated and their concentrations expressed as counts per minute (cpm). A cut off point of 718 cpm (based on the mean of the values obtained from 57 normal sera + 3 SD) was used to distinguish between positive and negative samples.

Statistical analysis

The data obtained from the three ELISAs and the RLA were quantitatively compared using cross tabulation tables and analysed using the κ statistics test.[15] A κ value between 0.8 and 1.0 denotes very good agreement between the assays, a value between 0.6 and 0.8 substantial agreement, and a value between 0.4 and 0.6 moderate agreement.[16]

RESULTS

Table 1 shows the individual data obtained using the four different assays. All 30 sera from the patients with AIH2 were positive by RLA, the in house ELISA, and the LKM1-ELISA, and 29 were positive by Varelisa.

Of the 55 LKM1 negative controls, all 55 were negative by in house ELISA and RLA; 54 were negative by Varelisa and 41 by LKM1-ELISA. The one sample positive by Varelisa was also weakly positive by LKM1-ELISA (12.5 AU/ml), but not by the other assays. This sample belonged to the child with AIH1 who, on one occasion five years previously, had been positive for LKM1 at a titre of 1/10. Of the 14 control samples positive by LKM1-ELISA, 11 were from patients with AIH1, two were from patients with Alagille syndrome, and one was from a child with α1ATD. When a cut off point of 18.74 AU/ml (the highest value observed in a normal subject) was used for LKM1-ELISA, the number of positive results in the control group decreased from 14 to two. These sera were from two patients with AIH1 and were negative by all other assays.

Table 2 shows the κ reliability test values. Agreement between immunofluorescence, in house ELISA, RLA, and Varelisa was high, whereas the agreement between immunofluorescence and LKM1-ELISA was moderate.

DISCUSSION

We found that one of the two commercial kits assessed in our study (Varelisa) allows the detection of LKM1 reactivity with a high degree of accuracy and that the other commercial kit (LKM1-ELISA) can also be of use clinically if its cut off point is readjusted.

Varelisa detected all but one of the sera that were positive for LKM1 by IFL and gave negative results in all but one of the LKM1 negative control sera. These results compare well with those obtained with the in house ELISA and RLA, showing 100% agreement with IFL. Interestingly, the only serum from the LKM1 negative group that was positive by Varelisa came from the patient with AIH1 who five years previously was positive for LKM1 by IFL at a low titre of 1/10 and for anti-CYP2D6 by RLA, although subsequent tests, including IFL, in house ELISA, and RLA, were negative. It is possible that this patient, although SMA positive, has low anti-CYP2D6 reactivity. Mixed autoimmune serology between AIH1 and AIH2 has been described previously.[17]

> "When access to a reference laboratory is impossible because of clinical urgency or when an interpretative doubt arises, these commercial assays provide the means for diagnosing or confirming with confidence anti-LKM1/CYP2D6 reactivity"

All 30 sera that were LKM1 positive by IFL, in house ELISA, and RLA were also positive using the LKM1-ELISA kit, but a quarter of the sera from the LKM1 negative control group were also found to be positive. As a consequence of this apparent higher sensitivity, the assay loses diagnostic specificity, because it also gives positive results in non-autoimmune disorders, such as α1ATD and Alagille syndrome. This high degree of positivity among LKM1 negative controls was seen when the cutoff value suggested by the manufacturer, calculated as 3 SD above the mean in a large population of healthy controls, was used. However, if only those values that

Take home messages

- All 30 sera that were liver kidney microsomal antibody type 1 (LKM1) positive by immunofluorescence were positive by the in house enzyme linked immunosorbent assay (ELISA), the radioligand assay, and LKM1-ELISA, and 29 were positive by Varelisa
- None of the 55 sera negative for LKM1 by immunofluorescence was positive by the in house ELISA and radioligand assay, but one was positive by Varelisa and 14 were positive using the LKM1-ELISA
- Thus, the Varelisa kit allows the accurate detection of LKM1 but a more clinically relevant cutoff point needs to be established before the LKM1-ELISA can be of diagnostic use

exceeded the highest result seen within the healthy population were considered to be positive, all the sera from the LKM1 positive patients, but only two from the LKM1 negative controls remained positive, suggesting that a more clinically relevant cutoff point needs to be established before the LKM1-ELISA can be of diagnostic use.

In the context of autoimmune liver disease, the detection of autoantibodies using indirect immunofluorescence on an appropriate multiorgan substrate remains the diagnostic mainstay, enabling the detection of multiple autoantibodies relevant to the diagnosis of autoimmune liver disease such as ANA, SMA, mitochondrial, liver kidney microsomal, liver cytosol type 1, and several other autoantibodies present in immunopathological conditions not affecting the liver. However, when access to a reference laboratory is impossible because of clinical urgency or when an interpretative doubt arises, these commercial assays provide the means for diagnosing or confirming with confidence anti-LKM1/CYP2D6 reactivity. This information is of crucial importance to the clinician, because AIH2, which is exquisitely responsive to immunosuppression, often presents as an acute hepatitis or even with fulminant hepatic failure.[17] The correct diagnosis will allow prompt treatment.

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