

Eosin-5'-Maleimide (EMA) Test for Hereditary Spherocytosis (Reference 2014.01.007)

Notice of Assessment

June 2014

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1 GENERAL INFORMATION

1.1 Requestor: CHU Sainte-Justine

1.2 Application for Review Submitted to MSSS: January 14, 2013

1.3 Application Received by INESSS: March 1, 2014

1.4 Notice Issued: June 30, 2014

Note

This notice is based on the scientific and commercial information submitted by the requestor and on a complementary review of the literature according to the data available at the time that this test was assessed by INESSS.

2 TECHNOLOGY, COMPANY AND LICENCE(S)

2.1 Name of the Technology

EMA test: flow cytometry test with eosin-5'-maleimide (EMA) staining for hereditary spherocytosis.

2.2 Brief Description of the Technology, and Clinical and Technical Specifications

This is a flow cytometry test (using the BD FACSCanto II analyzer) for measuring the fluorescence intensity of erythrocytes washed and incubated with a membrane-intercalating agent, eosin-5'-maleimide [Loosveld and Arnoux, 2010]. Fluorescence intensity is expressed as MFI (mean fluorescent intensity) units. Data are acquired using software [Do-Rouvière, 2008].

Eosin-5'-maleimide binds to erythrocyte membrane proteins, mainly band 3 protein. In cases of hereditary spherocytosis, elliptocytosis¹ and pyropoikilocytosis,² these proteins are missing and the resulting fluorescence is weaker [Mentzer, 2014].

EMA binding to lysine 430 in the first extracellular loop of the band 3 protein accounts for about 80% of the fluorescence. The remaining fluorescence is attributable to EMA binding to the sulfhydryl groups of the "Rh complex" (CD47, RhAG³ and Rh polypeptide) found on the surface of red blood cells. A decrease of these molecules on the surface of red blood cells has been shown in hereditary spherocytosis. EMA binding to these proteins (band 3, CD47, RhAG and Rh polypeptide), which are closely associated with the red cell cytoskeleton, underpins the high specificity of this test. Although EMA does not interact directly with spectrin, ankyrin or protein 4.2 — three molecules also associated with hereditary spherocytosis — EMA binding to CD47, Rh polypeptide and RhAG is an indirect but reliable marker of cytoskeleton composition [information provided by the requestor].

A decrease in fluorescence intensity is calculated as a percentage using the following formula (MFI controls – MFI patient)/MFI controls × 100 [Do-Rouvière, 2008].

The requestor interprets the results as follows:

¹ Erythrocytes are elliptical.

² Disease characterized by unstable cell membranes that makes the erythrocytes very fragile.

³ RhAG: Rhesus- or Rh-associated glycoprotein.

- 0% to 15% decrease in fluorescence (normal interindividual variability): normal, **negative for hereditary spherocytosis**;
- 15% to 20% decrease in fluorescence (slightly increased interindividual variability): suspect, **possible hereditary spherocytosis**;
- 20% or greater decrease in fluorescence (abnormally increased interindividual variability): abnormal, **probable hereditary spherocytosis**.

2.3 Company or Developer

Eosin-5'-maleimide (EMA) is manufactured by Molecular Probes®.

The analyzer used for flow cytometry is the BD FACSCanto II, manufactured by BD Biosciences®.

2.4 **Licence(s)**: Not applicable.

2.5 **Patent, If Any**: Not applicable.

2.6 **Approval Status (Health Canada, FDA)**: Not applicable.

2.7 **Weighted Value**: 62.16.

3 CLINICAL INDICATIONS, PRACTICE SETTINGS, AND TESTING PROCEDURES

3.1 Targeted Patient Group

Targeted patients are those in whom hereditary spherocytosis is suspected yet clinical diagnostic criteria have not been met and all other causes of hemolysis have been excluded.

3.2 Targeted Disease(s)

Hereditary spherocytosis (HS) or Minkowsky-Chauffard disease is the most common inherited hemolytic anemia in northern Europe and North America. Its incidence is about 1 in 5,000, but could be 1 in 2,000 if minor or subclinical forms are taken into account [Bolton-Maggs et al., 2011; Furs and Moriarty, 2009; Dhermy, 2006]. The hemolysis level is highly variable, ranging from an urgent need for transfusion to asymptomatic and discovered incidentally [Furs and Moriarty, 2009]. Transmission is autosomal dominant in 75% of cases; in others, it is caused by a *de novo* mutation or by a recessive autosomal inheritance [Dhermy, 2006]. Pathogenesis is related to the five cytoskeletal proteins of the erythrocytic membrane that regulate erythrocyte deformability and elasticity (α and β spectrin, ankyrin, band 3 protein and protein 4.2). Quantitative or qualitative abnormalities of these proteins are observed in patients with hereditary spherocytosis [Bolton-Maggs et al., 2011].

Diagnosis of hereditary spherocytosis is clinical, based on family history, typical clinical features (signs of anemia, splenomegaly, hyperbilirubinemia) and laboratory test results (presence of spherocytes, elevated mean corpuscular hemoglobin concentration and increase in reticulocytes). If the clinical information confirms the diagnosis, no additional tests are required. If the diagnosis is equivocal, other tests are necessary, such as cryohemolysis or EMA. These statements are graded recommendations (GRADE 1A) [Bolton-Maggs et al., 2011] (section 5.4).

Hereditary pyropoikilocytosis is a rare and severe form of elliptocytosis that affects black people.

3.3 Number of Patients Targeted

The requestor estimates that nearly 80 new tests will be performed annually.

3.4 Medical Specialities and Other Professions Involved

Hematology and pediatrics.

3.5 Testing Procedure

The analysis requires a whole blood sample (EDTA or sodium heparin) from the patient and two or three negative control samples (from a normal, unrelated, non-smoking patient) for each day of testing; it is important that the patient's samples and the control samples be drawn into the same anticoagulant [information provided by the requestor]. The age and sex of the patient and control must be indicated. Samples must be refrigerated (at 4°C, according to the requestor) and arrive at the laboratory within 96 hours (Mayo Medical Laboratories, HSEP test⁴).

4 TECHNOLOGY BACKGROUND

4.1 Nature of the Diagnostic Technology

Complementary test. According to the requestor, the EMA assay is a first-line test. If the result is inconclusive, the osmotic fragility test (OF) is performed (code 10070; WV: 139.0).

4.2 Brief Description of the Current Technological Context

There are several laboratory tests other than the EMA test for diagnosing erythrocyte membrane abnormalities. Among these first-line tests, we note in particular the OF, the acid glycerol lysis test (AGLT), the Pink test and cryohemolysis. Further investigation in uncertain cases includes SDS-PAGE,⁵ ektacytometry and molecular analysis [King and Zanella, 2013; King, 2011].

In a literature review published in 2013, King and Zanella briefly describe these various tests:

- Osmotic fragility: determines the concentration of NaCl producing 50% red blood cell lysis in fresh or incubated blood.
- AGLT and Pink test: determine the rate of lysis of erythrocytes suspended in a buffered glycerol solution. These tests do not differentiate HS from secondary spherocytosis associated with other diseases, especially autoimmune hemolytic anemias.
- Cryohemolysis: based on an increased susceptibility of spherocytes to rapid cooling from 37°C to 0°C in hypertonic conditions.
- EMA: has higher specificity than other tests because of its ability to bind dye to specific membrane molecules (band 3 macro complex).

King and Zanella [2013] report that the results of a survey by the European Network for Rare Red Cell Anemias (ENERCA) in 25 European reference centres show that 60% of the centres have adopted EMA and 50% use OF on fresh blood. Most of the centres use a combination of tests.

⁴ Mayo Medical Laboratories. Hereditary Spherocytosis Evaluation [website]. Available at: <http://www.mayomedicallaboratories.com/test-catalog/Overview/81087>.

⁵ SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) can be used to determine which protein is missing. Molecular diagnosis would be reserved for cases in which the phenotype among family members cannot be explained by the SDS-PAGE results. King [2011] indicates that it is not common practice because the genes associated with the affected protein are large, between 19 and 52 exons.

4.3 Brief Description of the Advantages Cited for the New Technology

According to the studies reported by Bolton-Maggs et al. [2011], EMA testing is easy to use, results are available in two to three hours, sensitivity and specificity are similar to the acidified glycerol lysis test and to ektacytometry and surpass those of the osmotic fragility test. The test can be done using a small volume of sample, which makes it suitable for children and neonates [King et al., 2000]. Other advantage of the test is that it can be done several days later (48 hours to 6 days) [Guitton et al., 2008].

When hereditary spherocytosis is suspected, a normal EMA test result can quickly rule out an HS diagnosis, which is worthy of interest, especially when laboratories do not have other, more complex technologies [Loosveld and Arnoux, 2010].

4.4 Cost of Technology and Options: Not assessed.

5 EVIDENCE

5.1 Clinical Relevance

5.1.1 Other Tests Replaced: None.

5.1.2 Diagnostic or Prognostic Value

This is a first-line test. When the decrease in fluorescence is between 16% and 21% (uncertain cases, possible diagnosis of hereditary spherocytosis), the investigation should be rounded out with another test, such as ektacytometry (EC) or SDS-PAGE. These two tests are not available everywhere, and the need for a rapid analysis after collecting the sample favours the EMA test [Girodon et al., 2008]. Although certain authors [King et al., 2000] show that this method is highly predictive for the diagnosis of hereditary spherocytosis, there is no correlation between the MFI values (mean fluorescent intensity) and disease severity.

5.1.3 Therapeutic Value

A rapid differential diagnosis of hereditary spherocytosis permits a better therapeutic approach, but there is no direct relationship between the test and treatment.

5.2 Clinical Validity

COMPONENT	PRESENCE	ABSENCE	NOT APPLICABLE
Sensitivity	X		
Specificity	X		
Positive predictive value (PPV)	X		
Negative predictive value (NPV)	X		
Likelihood ratio (LR)	X		
Receiver operating characteristics (ROC) curve	X		
Accuracy	X		

Table 1 presents the clinical performance of the EMA test results reported in 12 studies. This test's capacity for detecting hereditary spherocytosis is good in most of the studies, with sensitivity ranging from 89% to 100%, except in two studies that report sensitivities between 70% and 80% [Ciepiela et al., 2013; Crisp et al., 2011] and specificities ranging from 94% to 100%. The positive predictive values (PPV) and negative predictive values (NPV) reported in five studies range, respectively, from 81% to 97.8% and from 93% to 100%. According to the results of six studies, the area under curve ranges from 0.873 to 0.99. One study shows that the probability of the EMA having a positive diagnosis of hereditary spherocytosis (positive likelihood ratio or LR) is 18.0. However, the clinical utility of this test is lower because the negative LR is 0.31 [Crisp et al., 2011].

The cut-off values vary and are expressed in different ways; i.e., as a percent decrease of mean fluorescent intensity (11%, 17%, >17%, 18%, 19.5%, 21% and 80%), mean fluorescent intensity (0.80, 400 and 10,126 MFI) or MFI units (45.5, 48.2 and 91.5 MFI).

The question of cut-off value for mean fluorescent intensity decrease does not seem to be settled. Some authors report a grey area between 16% and 21% [Mackiewicz et al., 2012]. According to a study in France, reported in a letter to the editor, 85% of patients with hereditary spherocytosis showed a decrease in mean fluorescent intensity of over 15% and 134 controls had decreases of 15% or less [Mayeur-Rousse et al., 2012].

The diagnostic accuracy of the EMA test is >90%: 94.7% [Kar et al., 2010]; 96.91% [D'Alcamo et al., 2011] and 97.2% [King et al., 2000].

Table 1: Clinical validity of EMA for the diagnosis of hereditary spherocytosis

STUDY	NUMBER OF PATIENTS (FAMILIES)	CONTROLS	EMA CUT- OFF*	PERFORMANCE OF EMA				
				Se, %	Sp, %	AUC	PPV, %	NPV, %
Ciepiela et al., 2013 Poland	5	30 without HS	80%	80	100			
Simões et al., 2013 Portugal	115	441 healthy subjects	0.80 (MFI)	93	99.5			
Bianchi et al., 2012 Italy	150 (from 128 unrelated families)	575 healthy subjects	11%	93	98			
Crisp et al., 2012 Argentina	31 children (capillary blood) (age between 2 days and 7 years)		> 17%	90	-	-		
Crisp et al., 2011 Argentina	62 (55 with probable HS; 7 parents) from 4 families	263 blood donors	17%	70.2	96.1	0.873	81	93
D'Alcamo et al., 2011 Italy	33	130 healthy subjects	48.2% (MFI)	98.45	90.91	0.9927	93.75	97.69
Kar et al., 2010 India	114	20 healthy subjects	10,126 (MFI)	96.4	94.2	0.99	84.4	98.8
Loosveld and Arnoux, 2010 France	35	132 healthy subjects	18%	100	99.3			
Tachavanich et al., 2009 Thailand	50 (age between 2 weeks and 36 years)	142 healthy subjects	91.5% (MFI)	100	99.6	0.99		
Do-Rouvière, 2008 France	21 HS	29 without HS	19.5	100	93.1	0.95		
Girodon et al., 2008 France	53	6	> 21%	89	96			
Stoya et al., 2006 Germany	58 (from 40 families)	110 healthy subjects; 8 family members without HS	400 U (MFI)	96.6	99.1			
King et al., 2000 United Kingdom	98	180 healthy adults	45.5 U (MFI)	92.7	99.1	0.99	97.8	96.9

Abbreviations: AUC = area under curve; EMA = eosin-5'-maleimide; MFI = mean fluorescent intensity; Se = sensitivity; HS = hereditary spherocytosis; Sp = specificity; U = units

* Cut-off: MFI, percent decrease of MFI or MFI units.

The EMA test seems to be more sensitive than the osmotic fragility test. The comparison data are limited (Table 2).

Concomitant testing with two methods — the EMA test and the acidified glycerol lysis test (AGLT) — can diagnose 100% of patients with hereditary spherocytosis [Bianchi et al., 2012].

Table 2: Comparative performance of methods for diagnosing hereditary spherocytosis

STUDY	EMA			AGLT			OF			CH		
	Se, %	Sp, %	AUC	Se, %	Sp, %	AUC	Se, %	Sp, %	AUC	Se, %	Sp, %	AUC
Bianchi et al., 2012	93	98		95			68* 81†					
Crisp et al., 2012	90	-	-							95		
Crisp et al., 2011	70.2	96.1	0.873				58.8‡ 73.5§	99.1‡ 74.4§	0.784‡ 0.827§	78.7	95.8	0.916
Kar et al., 2010	96.4	94.2	0.99				71.4	74.1				

Abbreviations: AGLT = acidified glycerol lysis test; AUC = area under curve; CH = cryohemolysis; EMA = eosin-5'-maleimide; OF = osmotic fragility; Se = sensitivity; Sp = specificity.

* OF with NaCl — fresh

± OF with NaCl — incubated

‡ Incubated

§ Not incubated

5.3 Analytical (or Technical) Validity

COMPONENT	PRESENCE	ABSENCE	NOT APPLICABLE
Repeatability	X		
Reproducibility	X		
Analytical sensitivity		X	
Analytical specificity	X		
Matrix effect		X	
Concordance	X		
Correlation between test and comparator	X		
Other, depending on type of test		X	

Reproducibility

The reproducibility of results varies with the stability of the EMA solution, the concentration used, incubation time, sample storage conditions, and the time frame for analyzing the EMA-labelled erythrocytes [King et al., 2000].

Varies with length of storage

A rapid decrease in mean fluorescence is seen in the first three days when the EMA is stored at 4°C for one week. Four-month storage at -20°C also reduces mean fluorescence, although much less so [Cooper et al., 2007; Kedar et al., 2003; King et al., 2000].

Varies with delay between thawing and use

When EMA is thawed the day before its use, a fluorescence decrease of 40% to 60% has been observed on 6 normal control samples collected that day [Do-Rouvière, 2008].

Varies with the test sample storage time after erythrocyte labelling

Labelling red blood cells in a PBS–BSA⁶ solution after 48 hours storage at 4°C produces a stable MFI, as shown by the resulting coefficient of variation (CV) (between 0.6% and 2.6%) [Do-Rouvière, 2008]. Other authors show no difference between the percentages of fluorescence measured at 0 h (immediately after labelling), 1 h and 24 h of test sample storage at 4°C ($66.72 \pm 9.26\%$, $66.90 \pm 10.24\%$ and $67.86 \pm 11.31\%$, respectively) and no difference in MFI (26.44 ± 3.87 , 25.76 ± 3.97 and 26.88 ± 4.01 , respectively, *p* value not reported) [Ciepiela et al., 2013].

EMA concentration and incubation time

The recommended EMA concentration is 0.5 mg/mL, and the recommended incubation time is 1 hour.

Intra-individual variability

Test results are highly reproducible, with CV of 2.2% (12 control samples) [Do-Rouvière, 2008]. According to one study with repeated measurements of control samples, the CV was 2.25% for the positive controls and 2% for the negative controls [Kar et al., 2010].

Analytical specificity

Results are unaffected by a decrease in the mean corpuscular volume (MCV), as occurs in cases of iron deficiency anemia. However, fluorescence intensity is increased in higher MCV (e.g., in alcoholics) [King et al., 2000].

Concordance

The requestor carried out a concordance study between EMA and OF with 21 patients (1 control per patient). In 10 cases presenting abnormal EMA fluorescence (confirmed hereditary spherocytosis: HS+), FO increased; and in 7 cases presenting normal EMA fluorescence (negative for HS), FO was normal. In two cases considered “HS possible” based on EMA results, FO was slightly increased. There were two discordant cases: in one case the EMA result was “HS+”, and in the other it was “negative for HS”. Concordance was 90.5% (19/21) [information provided by the requestor].

- Correlation between test and comparator
- Correlation between EMA and AGLT: $r = 0.69$ [Stoya et al., 2006]
- Correlation between EMA and osmotic fragility: $r = 0.64$ [Do-Rouvière, 2008]

⁶ PBS–BSA = Phosphate buffered saline with bovine serum albumin.

5.4 Recommendations from Other Organizations

The Guidelines for the Diagnosis and Management of Hereditary Spherocytosis, issued by the British Committee for Standards in Haematology and updated in 2011 [Bolton-Maggs et al., 2011], recommends cryohemolysis and EMA tests for confirming cases of hereditary spherocytosis when the clinical diagnosis (family history, typical clinical features and usual laboratory investigations) is equivocal (GRADE 1 recommendation, GRADE A evidence).⁷ However, a study published as an abstract in 2013 [Park et al., 2013] challenges the performance of the cryohemolysis test and concludes that, despite the recommendation in these guidelines, the best tests for diagnosing HS are the EMA and FO tests.

According to recommendations from the German society for diagnosis and therapy of hematological and oncological diseases [Eber et al., 2012], no single test can identify all forms of hereditary spherocytosis, and for that reason it recommends using at least two different tests, preferably the EMA and AGLT tests. These ungraded recommendations are based on only one study [Bianchi et al., 2012]. In exceptional cases, after these two tests, the authors recommend ektacytometry, protein analysis or genetic analysis.

6 ANTICIPATED OUTCOMES OF INTRODUCING THE TEST

- 6.1 **Impact on Material and Human Resources:** Not assessed.
- 6.2 **Economic Consequences of Introducing Test Into Quebec's Health Care and Social Services System:** Not assessed.
- 6.3 **Main Organizational, Ethical, and Other (Social, Legal, Political) Issues:** Not assessed.

7 IN BRIEF

7.1 Clinical Relevance

The test is useful for diagnosing hereditary spherocytosis.

7.2 Clinical Validity

The clinical validity of EMA for confirming a hereditary spherocytosis diagnosis is well established, with overall sensitivity of 89% to 100% and specificity of 94% to 100%. However, considering the variability in fluorescence cut-off values used with the EMA test, as reported in the various studies, it is essential that all laboratories standardize their method and determine their reference cut-off value.

7.3 Analytical Validity

The reproducibility of the results obtained using EMA with flow cytometry depends on the stability of the EMA solution used. Few studies have been published on the topic, but available information indicates a CV of <3. One local validation study shows good concordance with the osmotic fragility test.

⁷ GRADE = Grading of Recommendations Assessment, Development and Evaluation: a GRADE 1 recommendation is a strong recommendation that can be applied to most patients; GRADE A evidence is high quality evidence: further research is unlikely to change confidence in the estimate of effect.

7.4 Recommendations from Other Organizations

The available recommendations are unanimous in stating that at least two different laboratory tests must be used when the clinical diagnosis of hereditary spherocytosis (family history, typical clinical features) and conventional laboratory investigations are inconclusive. EMA is a reliable method. The choice of other methods that could be used in combination with the EMA test is not established, and opinions differ.

8 INESSS NOTICE IN BRIEF

Eosin-5'-maleimide (EMA) test for hereditary spherocytosis

Status of the Diagnostic Technology:

- Established
- Innovative
- Experimental (for research purposes only)
- Replacement for technology _____, which becomes obsolete

INESSS Recommendation:

- Include test in the Index — conditional on the implementation of an external quality control mechanism in Quebec that will eventually include inter-laboratory exchange
- Do not include test in the Index
- Reassess test

Additional Recommendation:

- Draw connection with listing of drugs, if companion test
- Produce an optimal use manual
- Identify indicators, when monitoring is required

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