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Evaluation of sperm specific lactate dehydrogenase isoenzyme C4 (LDH C4) – Application to semen detection in stains

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Summary. The usefulness of LDH C (or LDH X) as a semen-specific marker has been tested. Many different methods have been applied for identification using various electrophoretic methods such as agarose gel electrophoresis, IEF, rocket-immunoelectrophoresis with subsequent enzymatical, immunological and immunochemical detection methods. Further methods applied were dot blot analysis for LDH C and PCR analysis for X/Y-chromosome differentiation. In blood/semen mixtures the LDH C-bands were detectable in dilutions up to 50-fold after staining for total LDH activity. An appropriate extraction procedure was developed. Immunological tests using a polyclonal antibody against LDH C were found to be highly specific and highly sensitive. The immunological test gave positive results with azoospermic stains up to a few weeks and in normal sperm stains positive reactions were obtained even after 8 months storage. A comparison of different detection methods for semen showed that immunological detection methods for LDH C are, at least after longer time periods of storage, superior to microscopical detection. – Swabs showed positive LDH C-reactions up to 48 h after intercourse. This reaction was closely correlated to PCR for male cells and to microscopical searching.

Key words: Semen detection – LDH C – Comparative study – Sensitivity and specificity – Body fluids

Zusammenfassung. Untersucht wurde die Anwendbarkeit des LDH C (oder LDH X) als spermien-spezifischer Marker. Zahlreiche Verfahren stehen für Identifikationsuntersuchungen unter Verwendung verschiedener elektrophoretischer Methoden wie Agarose-Gelelektrophorese, IEF, „Rocket-Immunoelektrophorese" in Kombination mit enzymatischen, immunologischen und immunochemischen Detektionsmethoden zur Verfügung. Weitere Methoden sind die Dot blot-Analyse für LDH C und die PCR-Analyse zur X/Y-Chromosom-Bestimmung. In Blut/Sperma-Mischungen sind die LDH C-Banden noch in bis zu 50-fachen Verdünnungen nach Anfärbung zur Bestimmung der LDH-Gesamtaktivität nachweisbar. Es wurde ein adäquates Extraktionsverfahren entwickelt. Immunologische Tests unter Verwendung von polyklonalen Antikörpern gegen LDH C waren hochspezifisch und sehr sensitiv. Die immunologischen Untersuchungen führten zu positiven Ergebnissen mit Spuren von azoospermischen Männern über einen Zeitraum von mehreren Wochen. Für spermienhaltige Spuren konnten bis zu einem Lagerungsalter von 8 Monaten positive Ergebnisse erzielt werden. Ein Vergleich über verschiedene Nachweismethoden für Spermien zeigte, daß – auch nach längeren Lagerungszeiträumen – die immunologischen Nachweismethoden für LDH C mikroskopischen Spermien-Nachweisen überlegen sind. Vaginalabstriche lieferten positive LDH C-Reaktion bis zu 48 Stunden nach Geschlechtsverkehr. Diese Ergebnisse waren eng korreliert zu PCR-Nachweisen der männlichen Zellen und zu mikroskopischen Untersuchungen.

Schlüsselwörter: Spermien-Nachweis – LDH C – Vergleichs-Untersuchungen – Sensitivität und Spezifität – Körperflüssigkeiten

Introduction

In forensic case work, the presence of semen can only be proven by the microscopic detection of spermatozoa or by the presence of other semen specific components such as LDH C4 (LDH-X) and p30 [4]. LDH C4 has only been found in testes, spermatozoa and seminal plasma and has never been observed in any other male or female tissues [14]. The occurrence of LDH C4 coincides with the onset of spermatogenesis and thus starts after puberty. LDH C4 activity accounts for approx. 80% of the total LDH activity in spermatozoa and 11% of LDH activity in human testes, and is present mainly in the mitochondria but partly also in the cytosol [6]. There are only a few papers dealing with LDH C4 investigation in forensic samples [3, 7–12]. Using conventional electrophoresis, LDH C4 has been detected in 4-week-old stains and

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it was suggested that the detection of the enzyme is sometimes superior to microscopical searching for the identification of semen [7, 9]. The presence of LDH C4 has also been investigated in vaginal, rectal and buccal swabs [8, 12]. In earlier investigations, we observed that electrophoretic detection of LDH C4 in seminal fluid and mixtures of semen with vaginal secretion is possible for at least 2 weeks [10].

The aim of this study was to analyse LDH C4 electrophoretically in semen stains, mixtures and vaginal swabs and to establish a reliable and sensitive method for semen detection which could be used as a supplement to microscopical searching.

Materials and methods

Semen samples were obtained from 120 males and the sperm count estiamted by electronic counting. Semen stains were made on cotton and stored at room temperature for 8 months. Residues of vaginal swabs taken after known time periods since last intercourse were obtained from the gynecology clinic. Semen samples were mixed with blood or saliva from female donors, dropped on cotton, and kept at room temperature.

Electrophoretic methods. Lactate dehydrogenase isoenzymes were separated in 1% agarose gel according to the method described by Shaler [12]. IEF of LDH isoenzymes was performed on ultra-thin (200 µm) polyacrylamide gel containing Ampholine pH 3.5–10 or pH 6–8 and 0.4 M beta alanin.

Detection of LDH and LDH C4 activity. Total activity of LDH in gel was detected with lactate as a substrate (0.2 mol/L) and 6 mmol/L NAD, 10 mmol/L MTT and 2.5 mmol/L PMS.

Immunoelectrochemical detection of LDH C4. Rocket immunoelectrophoresis was performed in 1% agarose gel [13] containing anti-human LDH C (kindly donated by Prof. E. Goldberg). After immunoeletrophoresis, immunoprecipitated LDH C was electrotransferred to the nitrocellulose membrane (NC) with 25 mM Tris HCl, 192 mM glycine pH 7.8 containing 0.1% SDS. The LDH C was specifically detected on NC with anit-human LDH C and the avidin-biotin detection system (Dakopatts, Denmark).

Dot blot analysis. LDH C4 from semen stains was extracted in buffer containing 100 mM Tris, 100 mM KCl, 1% Triton X-100, 100 µg/ml BSA and 5 mM DTT. NC membranes were blocked with 0.05% Tween 20 and subsequently incubated with rabbit anti-human LDH C diluted 1:8000. Avidin-biotin complex was used as a detection system.

PCR analysis. The presence of sperm and other male cells in vaginal swabs was analysed using X and Y chromosome specific primers flanking a segment of the amelogenin gene [1]. PCR products were electrophoresed on polyacrylamide gels according to the method of Allen and Budowle [2].

Results and discussion

LDH C-band detection and sensitivity and specificity. Semen specific LDH C band could easily be detected after simple agarose gel electrophoresis, isoelectric focusing (IEF) or IEF followed by immunoblotting. The IEF pattern from semen contained about 16 additional bands. This made it easily possible to detect semen in mixtures with other body fluids. For example, after IEF of blood/semen mixtures, the LDH C bands were detectable in dilutions up to 50-fold after staining for total LDH activity (Fig. 1). – After mixing with TBS they were even detectable in dilutions up to 75-fold. This difference in sensitivity might be due to a partial or complete disappearance of LDH C subunits from their specific sites since they can interact with the A and B subunits from blood leading to different electrophoretic mobility.

Extraction procedure

We have tested a variety of extraction procedures for semen stains on cotton stored for several weeks or months at room temperature. The conditions differed with regards to buffer compositions and extraction conditions [5] (data not shown). The best results were achieved after at least 12 h extraction at 4°C using Tris buffer pH 8.0, containing KCl, Triton X-100, BSA and DTT. – Special attention to the extraction procedure is necessary because LDH isozymes are tetramers and are easily susceptible to denaturation in stains. Extraction procedures should therefore aim at restoring the quarternary structure. In addition, DDT and Triton do not only help to refold the polypeptide chains and to reassociate LDH subunits, but also to liberate mitochondrial LDH C.

Dot blot analysis

Using the dot blot technique, as described, antibody dilution of 1:8000 was found to be optimal because semen

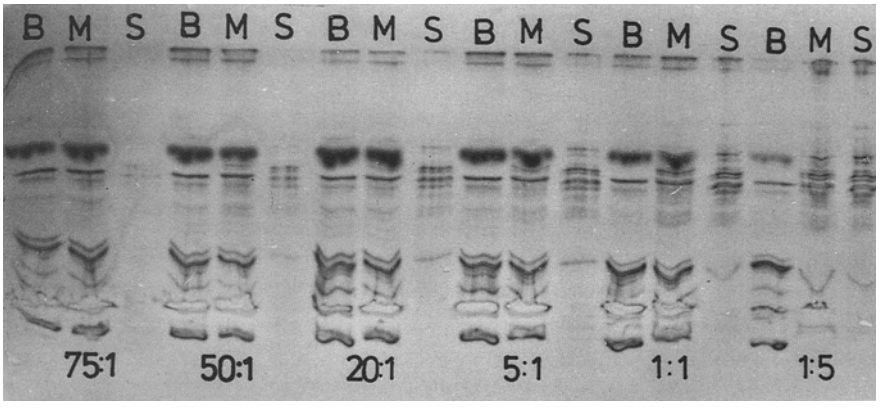

Fig. 1. IEF pattern of LDH isozymes from blood and semen separated in pH 3–10 polyacrylamide gel. Semen sample *(S)* and blood *(B)* were mixed *(M)* in different ratios (S:B 1:1–1:75) and subjected to IEF. LDH activity was detected using lactate staining

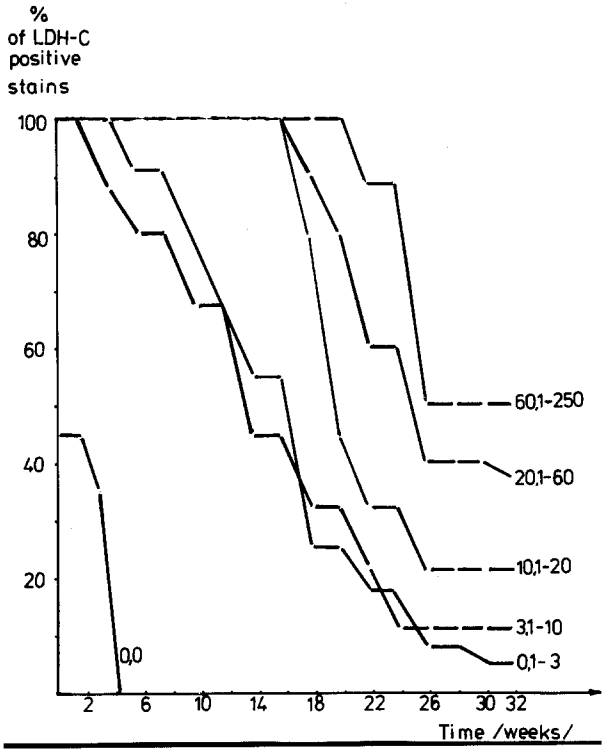

Fig. 2. Stability of LDH C in semen stains. Semen stains with different sperm counts ranging from 0–2.5 × 10^8/ml were stored at room temperature for 8 months. LDH C was analysed using dot blot method and avidin-biotin detection system

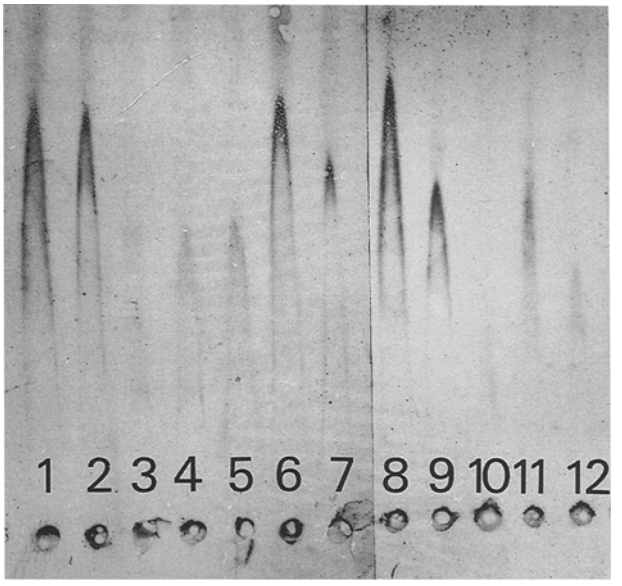

Fig. 3. Semiquantitative LDH C detection in semen stains using rocket immunoelectrophoresis with anti human LDL C antibody. "Rockets" represent LDH C precipitates from 6-month-old semen stains with sperm counts ranging from 0.2–2.5 × 10^8/ml as follows: 1) 1.74, 2) 1.51, 3) 0.43, 4) 0.72, 5) 0.80, 6) 1.96, 7) 0.89, 8) 2.50, 9) 0.91, 10) 0.21, 11) 0.69, 12) 0.52 (× 10^8/ml)

stains gave strong reactions and controls reacted negatively. The specificity has so far been tested with fresh blood samples and some body fluids (saliva, vaginal secretions, urine), which all gave negative results. Very weak positive reactions were observed in blood samples subjected to multiple freeze-thaw procedures. These were easily discernible from true 'positives', but special attention should be paid to mixtures of body fluids with substantial amounts of blood although the conditions described will be hardly reproduced in practice.

We have analyzed semen stains on cotton from 120 individuals with sperm counts ranging from 0 to 2 · 5 × 10^8 ml. These were stored at room temperature for up to 8 months: Most surprisingly, 45% of azoospermic stains (no spermatozoa present in the ejaculate) gave positive results up to 1 month (Fig. 2). 50% of stains with less than 10^7 ml spermatozoa reacted positively after 4 months (Fig. 2), and approximately 50% of stains with more than 2 × 10^7ml still reacted after 8 months. There exists an obvious correlation between time limits of reaction and the sperm count (Fig. 2).

LDH C concentration was assayed semi-quantitatively using rocket immunoelectrophoresis and immunoblotting. Since the Coomassie blue protein staining method is not sensitive enough, we used an immunoblotting system to detect immunoprecipitates of LHD C. The precipitated LDH C was electroblotted from agarose gel to NC, using transfer buffer supplemented with 0.1% SDS. Dissociated and transferred antigen-antibody complexes were subsequently detected on NC using the sensitive avidin-biotin system (Fig. 3). We did not observe any false positive reactions using this method.

Oya et al. [9] could demonstrate LDH-X using starch gel electrophoresis in normal semen stains up to 4 weeks old. Farriaux et al. [3] reported time limits of 35 days. Schmechta could detected LDH-X on cellulose membranes from stains up to 36 days old. In comparison to these and other earlier reports on LDH C stabilities in stains [3, 7–9, 11] we could detect LDH C in much older stains, in stains with very low sperm counts and partially also in azoospermic stains. This is mainly due to the use of more sensitive methods such as dot blot and rocket immunoelectrophoresis.

Comparative studies

A comparison of different LDH C detection methods tested for oligospermic semen stains is shown in Table 1. After 2 months storage at room temperature, 5 out of 20 samples gave negative results by microscopic searching. Analysis of LDH C in the same stains using 4 different methods showed that dot blot and rocket immunoelectrophoresis were superior to microscopy. Similiar observations have been made by Oya et al. [9]. Out of 38 case stains, 19 positive reactions were observed for LDH-X and spermatozoa, 4 were positive for spermatozoa and negative for LDH-X and 6 were LDH-X positive and spermatozoa negative. Mokashi and Madiwale [7], also observed that PAGE and LDH gave substantially better results than microscopic identification of spermatozoa in relatively old stains. From 25 stains more than 4 months old they obtained 12 positive for LDH-X and only 5 positives for microscopic detection of spermatozoa.

Table 1. Comparison of different LDH C detection methods for 2-month-old semen stains from oligospermic individuals

Storage time	Presence of sperm cells		Agarose gel electrophoresis		IEF		Rocket immuno-electrophoresis		Dot blot	
	+	−	+	−	+	−	+	−	+	−
1 day	20	0	20	0	20	0	20	0	20	0
2 months	15	5	2	17	12	8	18	2	19	1

+, number of positive results; −, number of negative results

Table 2. Detection of LDH C protein, spermatozoa and X and Y chromosomes specific sequences in vaginal swabs

Swab No	Time after intercourse	Number of spermatozoa	PCR analysis of sex specific sequences	LDH C dot blot
1	7 h	++++	X, Y	+
2	8 h	+++	X, Y	+
3	8 h	+++	X, Y	+
4	10.5 h	+++	X, Y	+
5	48 h	+++	X, Y	+
6	14 h	++	X, Y	+
7	7 h	+?	X, Y	−
8	14 days	−	X	−
9	2 days	−	X	−
10	7 days	−	X	−
11	14 days	−	X	−
12	21 days	−	X	−

Swabs

We also analyzed LDH C in vaginal swabs taken at known times after intercourse. Dot blot analysis was applied to detect LDH C. Swabs were also analyzed microscopically, and in addition PCR analysis was performed to detect the presence of male DNA (Table 2). In 6 cases Y chromosome-specific bands were observed. In these samples, spermatozoa and LDH C were also identified. Dot blot detection of LDH C showed positive reactions in 6 samples which were spermatozoa positive and Y band positive. In one sample (swab No. 7), with only 1 or 2 sperm cells on the microscopic slide, the PCR reaction was positive for male DNA and negative for LDH C. Because this swab was obtained 7 h after intercourse, and the number of spermatozoa was extremely low, with large amounts of bacteria, it can be assumed that the initial sperm count was low and LDH C was in addition rapidly destroyed by bacterial peptidases.

References

1. Akane A, Shiono H, Matsubara K, Nakahori Y, Seki S, Nagafuchi S, Yamada M, Nakagome Y (1991) Sex identification of forensic specimens by polymerase chain reaction (PCR): two alternative methods. Forensic Sci Int 49:81–88
2. Allen RC, Graves G, Budowle B (1989) Polymerase chain reaction amplification products separated on rehydratable polyacrylamide gels and stained with silver. BioTechniques 7:735–744
3. Farriaux J-P, Muller PH, Fontaine G (1967) Les isoenzymes de l'actiodehydrogenase du sperm human: Application medicolegale a l'identification d'une tache de sperme. Acta Med Leg Soc (Liège) 20:17
4. Gaensslen RE (1983) Sourcebook in forensic serology, immunology and biochemistry. National Institute of Justice, UD Government Printing Office, Washington DC
5. Gill P, Jeffreys AJ, Werrett DJ (1985) Forensic application of DNA "fingerprints". Nature 318:577–579
6. Goldberg E (1977) Isozymes in testes and spermatozoa. Isozymes. Curr Top Biol Med Res 1:79–124
7. Mokashi RH, Madiwale MS (1976) The use of sperm lactate dehydrogenase isoenzyme for the identification of semen in dried stains. Forensic Sci 8:269–275
8. Oya M, Kido A, Yada S (1977) Lactatdehydrogenase-Isozym X (LDH-X) als Hilfsmittel für den Nachweis von Ejakulatspuren in der Scheide. Arch Kiminol 160:115–118
9. Oya M, Kido A, Komatsu N, Kitahara N (1991) Zur praktischen Anwendbarkeit der LDH-X Bestimmung zum Nachweis von Spermaspuren. Arch Kriminol 187:91–95
10. Pawlowski R, Hauser R, Raszeja S (1988) Zur Stabilität ausgewählter Spermabestandteile in einem Gemisch von Sperma und Vaginalsekret. Beitr Gerichtl Med 46:219–226
11. Schmechta H (1975) Nachweisbarkeit und spurenkundlicher Wert der Isoenzyme der Laktatdehydrogenase bei der Untersuchung von Spuren. Dtsch Gesundheitswes 30:2443–2446
12. Shaler RC (1981) A multi-enzyme electrophoretic system for the identification of seminal fluid from postmortem specimens. Am J Forensic Med Pathol 2:315–321
13. Weeke B (1973) Rocket immunoelectrophoresis. In: Axelsen NH, Krolland J, Weeke B (eds) A manual of quantitative immunoelectrophoresis, pp 37–46
14. Wheat TE, Goldberg E (1983) Sperm-specific lactate dehydrogenase C4: antigenic structure and immunosuppresion of fertility. Isozymes. Curr Top Biol Med Res 7:113–130