TÉCNICA DO BIOSPECKLE LASER (BSL) NA AVALIAÇÃO DE SÊMEN BOVINO CONGELADO

PABLO HENRIQUE ANDRADE CARVALHO

2007

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Dissertação apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-graduação em Ciências Veterinárias, área de concentração em Fisiopatologia e Biotecnologia da Reprodução Animal, para a obtenção do título de "Mestre".

Orientador

Prof. Flamarion Tenório de Albuquerque

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APROVADA em 07 de Agosto de 2007.

Prof. Giovanni Francisco Rabelo	UFLA
Prof. João Bosco Barreto Filho	UFLA
Prof. Roberto Alves Braga Júnior	UFLA
Prof. Rubens Paes de Arruda	USP

Prof. Flamarion Tenório de Albuquerque
UFLA
(Orientador**)**

LAVRAS
MINAS GERAIS – BRASIL

“AQUELES QUE PASSAM POR NÓS NÃO VÃO SÓS.
DEIXAM UM POUCO DE SI, LEVAM UM POUCO DE NÓS”.

Antoine de Saint-Exupery

A DEUS, pela presença constante em minha vida,
AGRADEÇO

Aos meus pais Wander e Ronilda,
Meus irmãos Bruno e Diego,
Meus sobrinhos Gabriel e Giovana,
OFEREÇO.

À minha esposa Louiziane
e ao nosso bebê,
presente divino que está chegando,
DEDICO.

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LISTA DE ABREVIATURAS

PORTUGUÊS	ENGLISH
MI – momento de inércia	**IM** – inertial moment
Vig – vigor	**V** – velocity
Mot – motilidade individual	**PSCM** – percent sperm cell motility
CCM – concentração de células móveis	**MSCC** – mobile sperm cell concentration
CT – concentração total de células	**SCC** – sperm cell concentration
ML – microscopia de luz	**LM** – light microscopy
IVM – índice de vigor e motilidade	**VMI** – velocity and motility index
MASAC – método de análise de sêmen assistido por computador	**CASA** – computer-assisted semen analysis
MOC – matriz de ocorrências	**OCM –** occurrence matrix
sptz – espermatozóide (s)	**sptz** – spermatozoa
	BSL – bioepeckle laser
	CCD – charge coupled detector
	STS – spatial temporal speckle
	SQA – sperm quality analyzer
	SMI – sperm motility index
	SQI – sperm quality index

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CARVALHO, Pablo Henrique Andrade. **Técnica do *Biospeckle* Laser (BSL) na avaliação de sêmen bovino congelado.** 2007. 52 p. Dissertação (Mestrado em Ciências Veterinárias) – Universidade Federal de Lavras, Lavras, MG.[1]

A avaliação de viabilidade dos espermatozóides após o descongelamento é essencial para o controle de qualidade do sêmen. A técnica *Biospeckle laser* (BSL) já foi utilizada para mensurar atividade biológica em sementes, frutas, tecido embrionário e pode ser uma ferramenta para a análise de sêmen. Biospeckle é um fenômeno de interferência que ocorre quando um material biológico é iluminado por luz laser. Através do registro da história temporal do biospeckle é possível calcular o momento de inércia (MI), índice que quantifica a atividade do material analisado. Este trabalho apresenta resultados obtidos com a aplicação do BSL na avaliação de parâmetros de motilidade de sêmen bovino congelado. Em um primeiro experimento, propõe-se a técnica do BSL para a avaliação de vigor (Vig), motilidade individual (Mot) e concentração de células móveis (CCM) em sêmen bovino congelado. No segundo experimento, foram testadas duas diferentes fontes de luz laser para a análise de sêmen bovino com concentração padronizada. Um índice integrando Vig e Mot, avaliados microscopicamente, denominado índice de vigor e motilidade (IVM), foi proposto para comparação direta entre avaliação microscópica e o MI. A capacidade da técnica em detectar a degradação de Vig e Mot no período decorrido após o descongelamento foi investigada. Em ambas as etapas os valores de MI foram comparados com a avaliação microscópica, estabelecendo-se correlações entre os dois métodos. O sêmen foi diluído e congelado. Alíquotas de 10 µl foram utilizadas para a classificação de Vig, Mot, IVM, CCM e concentração espermática total (CT). Após a avaliação microscópica, cada amostra foi exposta à luz laser, originando imagens sucessivas do *biospeckle* que foram captadas por uma câmera e transformadas em uma matriz espaço por tempo, denominada *Spatial Temporal Speckle*, a partir da qual calculou-se o MI. Verificou-se que o MI tem correlação com os principais parâmetros da cinética espermática, refletindo a atividade dos espermatozóides, e que a técnica BSL representa uma ferramenta alternativa para a análise objetiva da qualidade de sêmen bovino congelado.

[1] Comitê Orientador: Flamarion Tenório de Albuquerque (Orientador), João Bosco Barreto Filho, Roberto Alves Braga Júnior (Co-orientadores).

ABSTRACT

CARVALHO, Pablo Henrique Andrade. **Bovine frozen semen under Biospeckle Laser (BSL) analysis.** 2007. 52 p. Dissertation (Master in Science, Animal Reproduction) – Federal University of Lavras, Lavras, MG.[1]

Sperm viability evaluation after thawing is essential to the semen quality control. Biospeckle laser (BSL) technique has been used to measure biological activity in seeds, fruits, embryonic tissue and could be an useful toll in semen evaluation. Biospeckle is an interference phenomenon which occurs naturally when a biologic material is illuminated by laser light. Through the record of the temporal history of biospeckle, it is possible to calculate the inertial moment (IM), index that quantifies the activity of the material submitted to the analysis. This work presents results obtained with the application of the BSL technique in the frozen bovine semen analysis, related to the spermatozoa kinetics aspects. In the first experiment, the BSL technique was investigated as an alternative method for the assessment of percent sperm cell motility (PSCM), velocity (V) and mobile sperm cells concentration (MSCC) in frozen bovine semen. In the second experiment, two different sources of laser light were tested for bovine semen samples evaluation with a standardized concentration. An index integrating V and PSCM, evaluated microscopically, named velocity and motility index (VMI), was proposed to compare light microscopy (LM) evaluation and the IM obtained by BSL. Thawed semen was evaluated by BSL in order to assess V and PSCM decreasing. In both experiments IM values were compared to LM evaluation and Spearman correlation test was used to verify association between the two methods. Semen was diluted and frozen. Aliquots of 10 µl were utilized for V, PSCM, VMI, sperm cell concentration (SCC) and MSCC evaluation. After LM evaluation, each sample was exposed to the laser light, giving rise to biospeckle images which were acquired by a camera and transformed into a time x space matrix, called the Spatial Temporal Speckle, from which IM was calculated. IM showed positive correlations with the major sperm kinetics parameters. Results suggest that BSL is an alternative and objetive method to frozen bovine semen analysis.

[1] Guidance Commitee: Flamarion Tenório de Albuquerque (Adviser), João Bosco Barreto Filho, Roberto Alves Braga Júnior (Co-adviser).

1 INTRODUÇÃO

Nas últimas décadas verificou-se uma expansão de técnicas aplicadas à reprodução animal. Dentre elas, a inseminação artificial (IA) assume atualmente posição de destaque na reprodução assistida, principalmente em bovinos. Neste contexto, a busca por métodos que avaliem o potencial de fertilidade do sêmen de reprodutores tem sido constante. Apesar de inúmeras técnicas desenvolvidas, ainda não existem métodos de avaliação aplicados *in vitro* que garantam a fertilidade do sêmen *in vivo*.

Dentre as características avaliadas rotineiramente no espermiograma, os aspectos de motilidade espermática são utilizados como principal critério para classificação, seleção e descarte de ejaculados nas centrais de IA.

Basicamente três parâmetros que se relacionam à cinética espermática são avaliados no exame andrológico: o turbilhonamento ou movimento de massa, presente no sêmen de ruminantes, caracterizado pelo movimento em forma de ondas de evolução dos espermatozóides, resultante de concentração e motilidade elevados. O vigor, ou intensidade de movimento, que influencia a velocidade dos espermatozóides; e a motilidade individual, que representa a proporção de espermatozóides móveis no ejaculado. Os dois primeiros parâmetros são avaliados em escalas de 1 a 5 e o terceiro em percentagem (%).

Apesar de ser uma característica de avaliação rápida, a estimativa da motilidade individual por microscopia de luz envolve um componente subjetivo que pode limitar a sua utilização. Em algumas espécies a literatura relata baixa correlação entre motilidade e fertilidade[1] e variações de 30 a 60% entre

[1] NIE, G. J.; WENZEL, J. G. W.; JOHNSON, K. E. Comparison of pregnancy outcome in mares among methods used to evaluate and select spermatozoa for insemination**. Animal Reproduction Science**, v. 69, p. 211-222, 2002.

observadores, para os mesmos ejaculados, foram verificadas quando se utilizou a microscopia de luz como método de avaliação[2].

Vários métodos de precisão, considerados objetivos, foram desenvolvidos com o intuito de se superar o problema da subjetividade na avaliação, tais como a fotomicrografia, técnica do *swin-up*, uso do funil ótico, migração dos espermatozóides em meio sintético e avaliação da cinética espermática por sistemas computadorizados.

O desenvolvimento de métodos de análise de sêmen assistidos por computador (MASAC) permitiu uma abordagem objetiva de diversas características das células espermáticas, como o padrão de motilidade, a velocidade de deslocamento e também a análise morfológica O principal problema, no entanto, está relacionado à padronização e otimização dos procedimentos e equipamentos.

Apesar das inúmeras aplicações e vantagens que estes métodos trazem para a análise de sêmen, sua utilização em Medicina Veterinária ainda é restrita e seu uso em maior freqüência concentra-se em clínicas de infertilidade humana. Porém, com o desenvolvimento e expansão de técnicas de reprodução assistida na Veterinária, como a inseminação artificial, transferência de embriões, fertilização *in vitro*, injeção intra-citoplasmática de espermatozóides e clonagem, a avaliação quantitativa e objetiva da cinética espermática se faz necessária.

A adoção de métodos ópticos e de processamento de imagens pode representar uma alternativa inovadora e acessível para a avaliação de qualidade de sêmen. Em publicações recentes são relatados resultados consistentes ao se utilizar o laser na avaliação da viabilidade e vigor de sementes[3] e qualidade de

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[2] IGUER-OUADA, M.; VERSTEGEN, J. P. Evaluation of the Hamilton-Thorn computer-based automated system for dog semen analysis. **Theriogenology**, v. 55, p. 733-749, 2001.

[3] BRAGA Jr, R.A.; DAL FABBRO, I. M.; BOREM, F. M.; RABELO, G. F.; ARIZAGA, R.; RABAL, H. J.; TRIVI, M. Assessment of seed viability by laser speckle techniques. **Biosystems Engineering**, v. **86**, p. 287-294, 2003.

frutos[4] apontando para a possibilidade de se utilizar luz laser na avaliação da qualidade de sêmen.

A luz laser (*Light Amplification by the Stimulated Emission Radiation*) é um tipo de radiação eletromagnética que apresenta um comprimento de onda bem definido, além de alta direcionalidade e coerência espacial e temporal[5]. As características especiais da luz laser têm despertado os pesquisadores para a potencialidade de sua aplicação em investigações científicas e na indústria.

A elevada intensidade e a grande direcionalidade da luz laser a torna interessante para um grande número de aplicações. Devido a essas características, quando um objeto é iluminado com a luz laser, ocorre a formação de uma figura de interferência devido ao espalhamento provocado pela constituição física da matéria desse objeto. Essa figura é denominada de *speckle* e sua aparência visual lembra o salpicado de uma mistura de grafite e sal sobre uma superfície. Quando o material iluminado apresenta algum tipo de movimento, ele se comporta como uma rede de difração dinâmica, o que irá provocar a mudança dessa figura ou padrão de interferência. Essa figura dinâmica lembra a tela de uma televisão fora de sintonia (chuvisco) e a ela, dá-se o nome de *biospeckle*, pois é característico principalmente da iluminação de tecidos biológicos com a luz laser, embora ocorra em fenômenos não biológicos.

Através da utilização de um detector de imagens, como uma câmara CCD (*Charge Coupled Detector*) é possível registrar essas mudanças na figura de interferência, permitindo-se assim, inferir sobre a dinâmica que ocorre no material sob estudo. Através da análise da variação temporal do *biospeckle* (padrão da figura de interferência) é possível detectar o movimento superficial, bem como movimentos internos do espécime iluminado. A análise estatística do

[4] RABELO, G. F.; BRAGA JÚNIOR, R. A.; FABBRO, I. M. D.; ARIZAGA, R.; RABAL, H. J.; TRIVI, M. R. Laser Speckle Techniques Applied To Study Quality Of Fruits**. Revista Brasileira de Engenharia Agrícola e Ambiental**, v. 9, p. 570-575, 2005.

[5] HECHT, E. **Optics**, 2. ed, New York: Adson Wesley, 1987, 676 p.

padrão do *speckle* possibilita atribuir um valor numérico que poderá ser correlacionado com o movimento detectado, tornando possível a diferenciação de espécimes com vários graus de movimentação[6], como é o caso dos parâmetros cinéticos do sêmen.

O equipamento utilizado para a aplicação da técnica *biospeckle* laser (BSL) consiste em uma fonte de luz laser de baixa potência, um expansor de feixes de luz, um espelho, uma câmera CCD e um microcomputador equipado com um processador de imagens. Um método utilizado para quantificar a atividade biológica do material em estudo é o momento de inércia (MI) apresentado por Arizaga e colaboradores em 1999[7].

O MI é um índice calculado a partir do de uma matriz denominada *Spatial Temporal Speckle* (STS), apresentada por Xu e colaboradores em 1995[8]. Para a formação do STS é necessário obter 512 imagens sucessivas do *biospeckle.* De cada imagem, uma linha é retirada, o que representa 512 pixels num mesmo instante e com as 512 linhas das 512 imagens forma-se o STS (512 x 512), onde as linhas representam a variação temporal de um pixel, e as colunas, a variação espacial em um mesmo instante.

O processamento do MI requer transformação do STS em um histograma bidimensional, denominado matriz de ocorrências (MOC), onde as linhas e as colunas representam intensidades luminosas (em tons de cinza) da imagem do STS. Desta forma, um algoritmo conta no STS o número de ocorrência (freqüência) que um pixel da imagem com determinado valor de intensidade

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[6] OULAMARA, A.; TRIBILLON, G.; DUVERNOY, J. Biological activity measurement on botanical specimen surface using temporal decorrelation effect of laser speckle, **Journal of Modern Optics**, v. 36, p. 165-179, 1989.

[7] ARIZAGA, R.; TRIVI, M.; RABAL, H.. Speckle time evolution characterization by co-occurrence matrix analysis. **Optics and laser technology**, v 31, p. 163-169, 1999.

[8] XU, Z. JOENATHAN, C.; KHORANA, B. M. Temporal and Spatial Proprieties of the Time-Varing Speckles of Botanical Specimens. **Optical Engineering**, v. 34, p. 1487 1495, 1995.

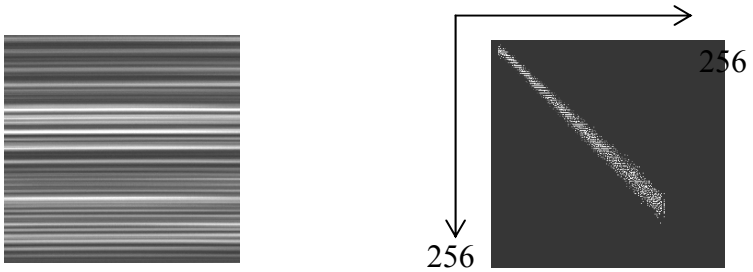
(linha) é seguido por de pixel que apresenta um outro valor de intensidade (coluna).

A MOC é definida na Equação 1, onde Nij representa o número de em que uma intensidade luminosa i foi seguida por uma intensidade luminosa j em duas imagens consecutivas do *biospeckle*.

$$MOC = \lfloor N_{ij} \rfloor$$

(Equação 1)

A Figura 1 mostra o STS formado após iluminação com luz laser de um material com baixa atividade biológica e a MOC correspondente. A Figura 2 ilustra o STS formado após iluminação com luz laser de um material com alta atividade biológica e a MOC correspondente.



(a) (b)

FIGURA 1: STS (a) e MOC (b) de um material com baixa atividade biológica.

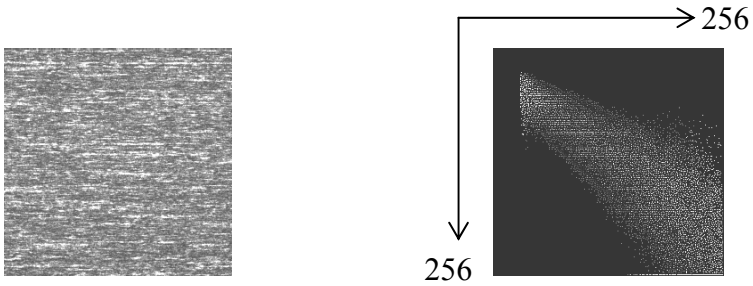


FIGURA 2: STS (a) MOC (b) de uma amostra com alta atividade biológica.

O MI é o somatório das freqüências ou ocorrências de uma intensidade i, seguido por uma intensidade j, multiplicada pelo quadrado da diferença de i menos j e representa, na realidade, a dispersão da MOC. Uma matriz muito dispersa, significa que ocorrem transições bruscas de intensidade no STS, gerando MI elevado. Por outro lado, uma matriz muito concentrada em torno de sua diagonal principal indica que ocorrem pequenas transições de intensidade na imagem do STS, gerando baixo MI.

A Equação 2 é define o cálculo do MI, obtido a partir do padrão STS, em que, i e j são coordenadas referentes às linhas e colunas do STS e N_{ij} é a freqüência ou número de ocorrências da intensidade i, seguida da intensidade j.

$$MI = \sum_{ij} N_{ij}(i-j)^2$$

(Equação 2)

Resultados preliminares dos Laboratórios de Óptica e Laser do Departamento de Engenharia e Reprodução Animal do Departamento de Medicina Veterinária da Universidade Federal de Lavras apontaram para a possibilidade de utilização do BSL na análise de motilidade e concentração

espermáticas em espécies de interesse na Medicina Veterinária e produção animal. No sêmen de cães, MI de 286,6 e 309,1 foram observados entre amostras com padrões próximos de motilidade e vigor. Já em garanhões, um deles com sêmen de motilidade conhecidamente inferior ao outro, pela análise convencional, observaram-se MI de 12 e 57, respectivamente.

Na espécie ovina, com a finalidade de se avaliar a sensibilidade do BSL a diferentes padrões de motilidade, em um mesmo indivíduo, um ejaculado foi submetido a choques térmicos sucessivos, realizando-se leituras consecutivas do *biospeckle.* Logo após a colheita o MI gerado foi de 700 e turbilhonamento avaliado em 3. A mesma amostra vinte minutos após a colheita, com atividade deprimida, apresentou MI de 133 e, após dois choques térmicos consecutivos, os valores de MI caíram para 73 e 23, respectivamente. A partir destes resultados vislumbrou-se a possibilidade de aplicação da técnica BSL para a avaliação de sêmen em outras espécies. Neste contexto, este estudo vem contribuir para o desenvolvimento de uma nova técnica de análise laboratorial para a avaliação da qualidade de sêmen bovino congelado.

Neste trabalho serão apresentados dois artigos. No primeiro, sêmen bovino congelado foi avaliado pelo BSL com a finalidade de investigar a sensibilidade da técnica em relação a diferentes padrões de motilidade individual, vigor e concentração de células móveis, bem como a influência destas características sobre os valores de MI, em diversas amostras de sêmen.

No segundo artigo, após a constatação de que o MI foi correlacionado estatisticamente com os principais parâmetros de motilidade espermática, o BSL foi testado sob condições específicas. Duas fontes distintas de laser foram utilizadas para a avaliação de amostras de sêmen bovino, congeladas com concentração padronizada. Um índice integrando vigor e motilidade, avaliados microscopicamente, denominado índice de vigor e motilidade, foi proposto como forma de comparação direta entre a avaliação por microscopia de luz e o

MI. O BSL foi testado para detectar a degradação de vigor e motilidade em função do tempo decorrido pós-descongelamento das amostras de sêmen.

2. ORIGINAL PAPER I

Motility Parameters Assessment of Bovine Frozen Semen by the Biospeckle Laser (BSL)

(Preparado de acordo com as normas da revista
"Reproduction in Domestic Animals")

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2.1 Contents

Motility evaluation is a necessary step in semen analysis by artificial insemination centers, once mobility can be related to sperm fertility; a widespread method to evaluate spermatozoa progressive movement and velocity is the light microscopy. Limitations of the routine methods unfolded new approaches of evaluations mainly in optical branches. The incidence of laser light in specimens exhibiting biological activity generates an interference image named biospeckle which has been used to investigate biological activity such as in plants, animals and human beings. The aim of this work was to evaluate frozen semen kinetics of bovine species, comparing light microscopy with biospeckle analysis. Thirty straws were thawed and semen was illuminated by Helium-Neonium laser just after light microscopy evaluation. Biospeckle images were processed using a second order statistical approach obtaining a summary value named inertial moment. Data were statistically analyzed using Spearman correlation and box-plots. Inertial moment approach was correlated (r = 0.904)

to sperm cell velocity ($p < 0.0001$). In addition, percent sperm cell motility and mobile sperm cell concentration presented positive correlation ($r = 0.977$; $r = 0.815$; respectively, $p < 0.0001$), evidencing coherence between semen activity features and inertial moment values. Sperm cell concentration did not influenced inertial moment values ($r = 0.262$; $p > 0.05$). These data indicate that biopeckle laser should be considered a reliable tool for semen motility assessment contributing as an alternative method to the sperm cell fertility investigation.

2.2 Introduction

Semen manipulation, dilution and freezing cause serious damages to the sperm cell, decreasing motility and affecting the membranes integrity (Hammerstedt et al. 1990). These damages and the standardization of viable sperm cells by the artificial insemination centers, for commercial purposes, make post-thawing evaluation a routine procedure to determine semen quality.

Progressive linear motility assessment by light microscopy (LM) is the standard method for classification, selection and disposal of ejaculates which are destined for thawing and commercialization in the artificial insemination industry (Nie et al. 2002). Nevertheless, this method is highly subjective and is influenced by the technician ability, training and experience. Differences ranging from 30 to 60% to the same ejaculate were observed among technicians when LM was used (Iguer-Ouada and Verstegen 2001). Besides, for some species, data have shown a weak correlation between motility evaluated by LM and fertility (Nie et al. 2002).

The biospeckle laser (BSL) consists of a laser beam illumination upon biological samples and the signal produced on images was used to investigate blood flow (Briers 1975), parasites (Romero 1999), seed analysis (Braga et al. 2003), to discriminate living and dead tissues (Rodrigues 2003), post-harvesting changes in oranges (Rabelo et al. 2005) and to detect fungi in seeds (Braga et al.

2005). Images processed by the BSL generate an index called inertial moment (IM), which quantifies the biological activity provenient from the light scattering produced by the illuminated specimen (Arizaga et al. 1999).

Preliminary data obtained in this laboratory (unpublished data) with equine, ovine and the dog *in natura* ejaculates had pointed out to the possibility of BSL use in semen analysis. IM values were positively correlated with the major sperm kinetics parameters, e.g., velocity, percent and gross motility. The interpretation of functions on light scattered from the bull motile spermatozoa requires models which consider the size, shape, and unusual swimming trajectories of these cells (Harvey and Woolford 1980).

The goal of this work was to evaluate bull frozen semen motility (velocity and percent sperm cell motility) and concentration (sperm cell concentration and mobile sperm cell concentration), comparing the IM generated by the BSL with LM evaluation, in order to verify whether spermatozoa numbers and motility could be assessed by this optical assay.

2.3 Material and Methods

2.3.1 Frozen semen samples

Semen was diluted in egg-yolk extender (lactose 7.4 nmol x L^{-1}; egg-yolk 20% - v/v; glicerine 7%v/v; added with 10^5 IU peniciline K and 10^5 µg dihidrostreptomicine) cooled at 5^{o} C for approximately 90 minutes, filled in thirty plastic straws with 0.5 milliliters capacity (IMV technologies, France) and after equilibration for 4 hours at 5^{o}C, it was frozen according to Nagase and Niwa procedures (1964). Straws were kept in liquid nitrogen (-196^{o} C) until evaluation.

2.3.2 Semen thawing

Straws were thawed at 37º C for 30 seconds, then the semen was introduced in 1.5 ml eppendorf microtubes and maintained at 37º C in a water bath until light microscopy evaluation.

2.3.3 Semen analysis and physical parameters evaluation

Motility and concentration of the semen samples were analyzed according to the Brazilian College of Animal Reproduction guidelines (Henry and Neves 1998).

Velocity (V) evaluation was performed using LM when 10 µl semen aliquots were pre-warmed at 37º C, covered with a slip and analyzed at 200 X magnification. Estimation was made in a 0 to 5 scale. Percent sperm cell motility (PSCM) was evaluated under the same conditions, using 200 X and 400 X magnifications.

Sperm cell concentration (SCC) was measured in a haemocytometer, where a sample of phormol-saline buffered solution diluted semen (1:50 ratio) was placed. Sperm cells were counted in 25 squares, in both sides of the chamber. Mobile sperm cell concentration (MSCC) was estimated by PSMC x SCC product.

2.3.4 BSL semen evaluation

The semen samples were illuminated by Helium-Neonium non-polarized laser light (632 nm and 10 mW) after 30 seconds of stabilization, immediately after LM evaluation, using the same slides prepared to microscopic analysis. Images were acquired by a CCD and analyzed in computer adopting approaches suggested by Oulamara et al. (1989), Xu et al. (1995) and Arizaga et al. (1999). Analyses were conducted building Space Time Speckle (STS) matrices from centre columns of 512 successive images of the dynamic speckle.

STS matrices were processed implementing a second order statistical analysis creating, in the middle step, an occurrence matrix (OCM), as presented in Eq.1.

$$OCM = \lfloor N_{ij} \rfloor \text{ (Eq.1)}$$

where Nij is the number of occurrences of successive intensity values i,j.

The entries of the OCM are the number of occurrences of a certain intensity value *i* followed by an intensity value *j*, which accordingly to Arizaga et al. (1999) characterize a particular case of the spatial gray level dependence matrix, employed to characterize the image texture. OCM matrix should be normalized, and the normalization adopted in this work was the division of whole matrix by 256, which represents the highest value of gray level.

A summary of the OCM can be created by measuring the spread of the N values around its main diagonal, presented in the Eq. 2. This second order moment is then called inertial moment (IM) of the matrix with respect to its main diagonal. Further details about that un-dimensional number can be seen in Arizaga et al. (1999).

$$IM = \sum_{ij} N_{ij} (i - j)^2 \text{ (Eq. 2)}$$

Biospeckle laser analysis was done at room temperature, in the absence of light, movements or noise inside the laboratory. Semen samples were illuminated by the laser light for 40 seconds (s) representing the time needed to get 512 images under a time rate of 0.08s.

2.3.5 Statistical analysis

Inertial moment (IM) data, for combinations among V (Velocity), PSCM (Percent sperm cell motility) and MSCC (Mobile sperm cell

concentration) classes were evaluated by an exploratory analysis, comparing mean and standard deviation and using box plots to show data dispersion. Correlation coefficients among V, PSCM, SCC, MSCC and IM were obtained and tested by the Spearman correlation test, with a significance nominal level of 5%. For all statistical analysis the R software (version 2.5.0) was used (R 2007).

2.4 Results

Inertial moment obtained by the BSL was positively correlated with the major semen physical parameters evaluated by light microscopy, as shown in Table 1. Velocity, PSCM and MSCC exhibited correlation coefficients greater than 0.815 in relation to IM ($p < 0.001$), indicating high association among these variables. However, between sperm cell concentration and IM no correlation was found ($p = 0.161$).

2.4.1 Inertial moment related to velocity and percent sperm cell motility

The relations among IM values, V and PSCM are presented in Table 2. Between V and IM correlation coefficient was 0.904 ($p < 0.0001$). IM values increased and the greatest mean (469.5 ± 118.4) was observed when velocity score was the higher ($V = 4$).

The box-plot (Figure 1) shows the IM data behavior when percent cell motility and velocity score were grouped in classes together. IM increasing values were observed in V x PSCM higher combination classes (C44, e.g., $V = 4$ and $PSCM = 4$, to C55). IM values greater than 400 were observed in classes showing V equal to 4 and 5 and PSCM higher than 55%. On the other hand, semen samples exhibiting PSCM lower than 20% and within $1 \leq V \leq 2$ showed IM lower than 200.

2.4.2 Inertial moment related to velocity, percent sperm cell motility and mobile sperm cell concentration

When percent sperm cell motility was considered, in relation to IM, a positive correlation was verified ($p < 0.001$; $r = 0.815$) despite of the fact that the total sperm cell concentration (live and dead cells) did not show significant results. The Tables 3 and 4 show IM average values in relation to V, PSCM and MSCC.

The Figures 2 and 3 are box plots of Tables 3 and 4 data, grouped according to the combination classes MSCC x V and MSCC x PSCM, respectively. In the same MSCC class, when V increases IM means also increase. In the same V class (V = 5) it was observed that samples with increasing MSCC exhibited IM higher means, but in samples showing velocity scores from 2 to 4 it did not happen.

In the same MSCC class it was verified that IM means increase when PSCM increase. In semen samples exhibiting PSCM over 75% (class 6) higher concentrations showed higher IM means. However, motility classes from 1 to 5 did not follow this pattern.

Inertial moment data presented low variation in 1 to 4 MSCC classes and in the 1 to 5 PSCM classes. IM higher standard deviation was observed in samples that MSCC ranged from 200 to 250 x 10^6 sperm x ml^{-1} and PSCM $\geq$ 75%. This pattern was observed for all parameters compared by LM and BSL.

2.5 Discussion

2.5.1 Inertial moment and the sperm cell concentration

Among all samples the sperm cell concentration ranged from 10 to 500 x 10^6 sperm cells x ml^{-1} and no correlation was observed between the inertial moment and SCC. Thus, dead or immobile sperm cells in thawed semen did not

influence the dynamic speckle or IM results. Preliminary data of this laboratory (Carvalho et al. 2005) showed that in the ram semen, with SCC ranging from 1.42 to 5.16 x 10^9 sperm cells x ml^{-1}, IM values were not influenced by the immobile cells in the ejaculate. Light scattering by the dead sperm cells is identified by the doppler (Ross et al. 1983) and influence optical methods using this principle. It seems that the BSL is not affected by these cells but by spermatozoa velocity, percentage and concentration of motile cells and probably by the movement pattern, identifying only viable gametes.

Woolford and Harvey (1982) pointed to the importance of immotile cells presence in the ejaculate, interactions or collisions between motile and immotile cells, the distribution of spermatozoa within the scattering cell, spatial orientation effects and the shape of the bull spermatozoon to understand the spectrum or autocorrelation function of the scattered light by semen. High IM values are observed when components of the illuminated specimen show abrupt changes in the gray level values between two subsequent images in the STS images, what is observed in high activity biological samples. Therefore, in semen analysis, it seems that IM is more influenced by the fast moving sperm cells, showing drastic position changes, rather than by dead sperm cells, passively moving in a slow and continuous flux in the ejaculate or extended semen. IM method concept allows a strict evaluation about what sort of information will be considered in its results. The concept is based in the ability of the method to follow abrupt changes in the material which are expressed in the data analyzed as high frequencies components, whilst the low frequencies in the signal analyzed will not contribute expressively in the IM values. That assumption correlates the activity of living cells with the IM results being the slow information from dead or weak spermatozoa damped by the technique.

In the broiler, when SCC was 0.1 to 6.0 x 10^9 sperm cells x ml^{-1}, using semen extended in 2, 3, 5, 9, 13, 17, 21 and 25 ratio and evaluated by the Sperm

Quality Analyzer (SQA), McDaniel et al. (1998) verified that SCC influenced the Sperm Motility Index (SMI). The highest SMI value (439) was observed in a semen sample showing SCC of 1.0 x 10^9 sperm cells x ml^{-1}. *In natura* semen samples exhibited low SMI values (36-85). However, SMI increased when semen was extended three fold and reached the highest value (439) in 1:5 dilutions. SMI decreased in dilutions from 5 to 25 fold, showing values under 200.

Bovine head sperm area shows, in average, 40.49 μm^2, measured by computer assisted semen analysis (Boersma et al. 1999) and the middle piece length is about 12 to 13 μm (Barbosa 1996). The sperm head is the major light scattering source of the cell, because its area is greater than the tail (Woolford and Harvey 1980). It is necessary, so far, to consider that the dimension differences between the bull and broiler sperm cell should be related to the different analysis patterns presented by BSL and the SQA, when SCC is under evaluation. To the broiler SQA semen analysis, it is possible that a minimum sperm cell numbers is necessary to be detected by the technique.

Palmer and Barth (2003) using the BullMate^{TM} SQA, that generates the Sperm Quality Index (SQI), which represents motility, concentration, viability and sperm morphology, observed that the MSCC ($r = 0.82$) and SCC ($r = 0.80$) were positively correlated when evaluating bull ejaculates and comparing with LM technique.

2.5.2 Inertial moment, sperm cell motility and velocity

Between velocity and IM correlation coefficient was 0.904 ($p < 0.0001$). IM values increased and the greatest mean (469.5 $\pm$ 118.4) was observed when velocity score was the higher ($V = 4$). Previous work (Carvalho et al. 2005) has found correlation of 0.7210 between velocity and e IM ($p < 0.0001$) in the ram semen evaluation by the BSL. In the same work, IM mean values ranged from

417 to 1787. One way to explain such distinct IM values between ram and bovine semen could be the sperm cell velocity. While the sperm cell average velocity in the ram semen is 100 to 150 μm x s^{-1} (Arman et al. 2006), in the bull frozen semen the average velocity is about 90 μm x s^{-1}.

Inertial moment is capable to distinguish different movement intensities, which is considered to be an important component to sperm viability and fertility. McDaniel et al. (1998) observed correlation between the SMI and the motility scoring method. This method reveals SMI dependence on the sperm cell activity in a semen sample. It seems that SMI, like the IM, reflects not only the number of sperm cells moving in a semen sample but also the intensity of sperm motility. Johnston et al. (1995) and Mahmoud et al. (1998) observed that SMI values were correlated to SCC, PSCM and sperm morphology, for bovine and *in natura* human semen, respectively. It appears that the inertial moment is insensible to detect immotile cells, but possibly it is related to sperm morphology, considering the different IM values observed among species showing different sperm dimensions. Experiments are being carried out to verify this hypothesis.

A high correlation coefficient (0.997) was observed between PSCM and IM ($p < 0.001$). The laser light scattering is related to the sperm spin movement and to an optimum (perpendicular direction to the scattering vector) head reflection angle (Harvey and Woolford 1980). A moving cell shows this angle various times along its trajectory in relation to dead cells, moving passively in the ejaculate. IM higher values observed in samples exhibiting high PSCM should be related to this fact.

In this experiment, among samples with PSCM above 55% (classes 1, 2, 3, 4 and 5) IM data showed little variation and dispersion. However, in the C65 class IM outliers were observed. It seems that when semen activity is very high,

BSL sensibility decreases. It has been previously observed in the ram semen (unpublished data) when gross motility estimate was $\geq$ 4 and velocity = 5.

Considering light microscopy analysis, it seems that human sight is able to distinguish limits among V classes varying from 1 to 3 and PSCM $\leq$ 50%. However, when the sperm cell activity increases it becomes more difficult to differentiate limits among velocity classes varying from 3 to 5 and PMSC $\geq$ 50%. Moreover, it should be considered that another BSL set up must be achieved to analyze semen in such situations. For example, Makler et al. (1999) did not find similarity between SQA II data and LM. Even in the CASA systems, there are extrinsic factors to the sample that must be controlled to have results with good precision, like extender quality and set up configuration (Anzar et al. 1991).

Sperm cell viability is a complex phenomenon and motility is just one of its components. According to Amann and Hammerstedt (1993) few isolated parameters related to viability show significant correlations with thawed semen fertility, mainly when the motility is high

Despite all the efforts and advances in the sperm morphology and viability investigation, we are not able yet to predict spermatozoa fertility (Rodríguez-Martinez 2006). The inertial moment generated by the BSL was correlated with the major bovine frozen semen motility parameters, grouping them in a single index, and it is possible that it could indirectly evaluate sperm morphology, giving a general estimate of semen quality and viability. Data of this work indicate that the BSL should be considered an alternative and useful tool for bovine frozen semen motility assessment and can contribute to the sperm cell fertility investigation.

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2.7 Tables

TABLE 1 – Spearman correlation coefficients estimates (n = 30 semen samples), with respective significances, to variables velocity (V), percent sperm cell motility (PSCM), mobile sperm cell concentration (MSCC), sperm cell concentration (SCC) and inertial moment (IM) in bovine frozen semen.

	V	*PSCM*	*MSCC*	*SCC*	*IM*
V	1.000	0.933 (p<0.001)	0.857 (p<0.001)	0.388 (p=0.034)	0.904 (p<0.001)
PSCM	0.933 (p<0.001)	1.000	0.863 (p<0.001)	0.319 (p=0.086)	0.977 (p<0.001)
MSCC	0.857 (p<0.001)	0.863 (p<0.001)	1.000	0.693 (p<0.001)	0.815 (p<0.001)
SCC	0.388 (p=0.034)	0.319 (p=0.086)	0.693 (p<0.001)	1.000	0.262 (p=0.161)
IM	0.904 (p<0.001)	0.977 (p<0.001)	0.815 (p<0.001)	0.262 (p=0.161)	1.000

TABLE 2 – Inertial moment (IM) mean values (standard deviation) related to velocity and percent sperm cell motility (PSCM) in bovine frozen semen.

		PSCM						Mean
		5	10	20	40	55	75	
Velocity	0.5	66.9 (11.4)	----	----	----	----	----	66.9 (11.4)
	1	----	134.8 (28.4)	----	----	----	----	134.8 (28.4)
	2	----	----	203.3 (30.9)	----	----	----	203.3 (30.9)
	3	----	----	----	316.9 (0.8)	450.5 (30.3)	----	397.0 (76.2)
	4	----	----	----	332.2 (50.8)	447.9 (10.8)	560.6 (74.0)	469.5 (118.4)
Mean		66.9 (11.4)	134.8 (28.4)	203.3 (30.9)	326.1 (36.9)	449.5 (22.1)	560.6 (74.0)	

TABLE 3 – Inertial moment (IM) mean values (standard deviation) observed according to mobile sperm cell concentration (MSCC) and velocity in bovine frozen semen.

		Velocity					Mean
		0,5	1	2	3	4	
MSCC (sptz x 10^6 ml^{-1})	< 50	66.9 (11.4)	146.1 (29.6)	191.1 (31.9)	----	----	125.5 (54.2)
	50 a 100	----	115.9 (14.9)	227.8	458.2	440.3	245.7 (163.9)
	100 a 150	----	----	----	316.9 (0.8)	347.8 (60.9)	332.3 (39.4)
	150 a 200	----	----	----	446.6 (41.8)	507.6 (38.2)	487.2 (47.1)
	200 a 250	----	----	----	----	509.7 (196.1)	509.8 (196.0)
Mean		66.9 (11.4)	134.8 (28.4)	203.3 (30.9)	397.0 (76.2)	469.5 (118.4)	

TABLE 4 – Inertial moment (IM) mean values (standard deviation) related to mobile sperm cell concentration (MSCC) and percent sperm cell motility (PSCM) in bovine frozen semen.

		PSCM						Mean
		5	10	20	40	55	75	
MSCC (sptz x 10^6 ml^{-1})	< 50	66.9 (11.4)	146.9 (29.6)	191.1 (31.9)	----	----	----	125.5 (54.2)
	50 a 100	----	115.9 (14.9)	227.8	----	449.2	----	245.7 (163.9)
	100 a 150	----	----	----	332.3 (39.4)	----	----	332.3 (39.4)
	150 a 200	----	----	----	----	449.6 (29.9)	524.9 (19.7)	487.2 (47.1)
	200 a 250	----	----	----	301.1	----	614.1 (107.6)	509.8 (196.0)
Mean		66.9 (11.4)	134.8 (28.4)	203.3 (30.9)	326.1 (36.9)	449.5 (22.1)	560.6 (74.0)	

2.8 Figures

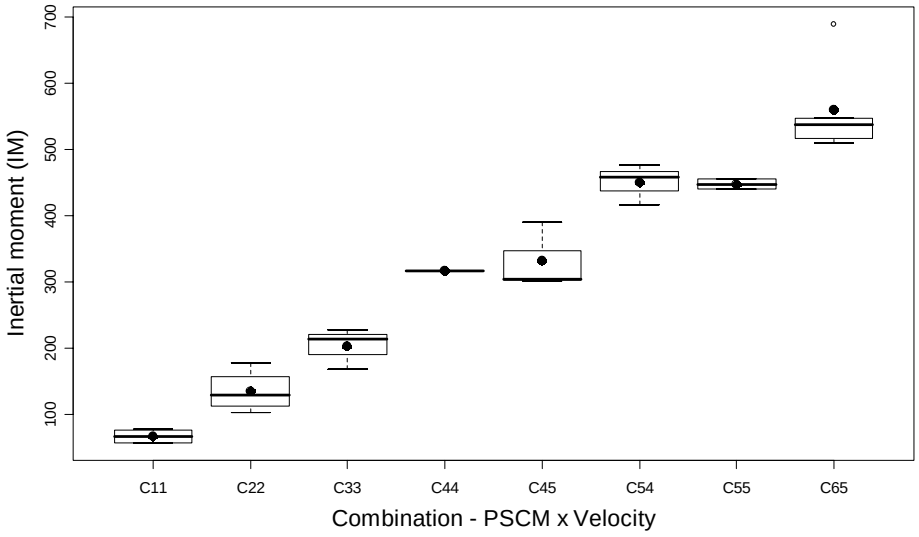

FIGURE 1- Box plot to inertial moment (IM) values, according to the combinations of percent sperm cell motility (PSCM) classes (class 1 = 5%, 2 = 10%, 3 = 20%, 4 = 40%, 5 = 55% e 6 = 75%) and velocity (class 1 = 0.5, 2 = 1, 3 = 2, 4 = 3 e 5 = 4) of bovine frozen semen.

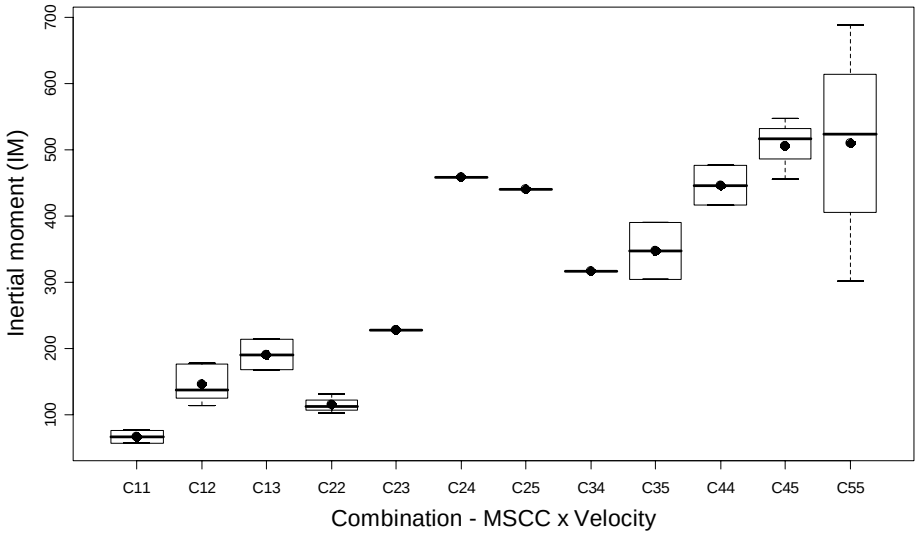

FIGURE 2- Box plot for inertial moment (IM) data values, according to the combinations of mobile sperm cell concentration (MSCC) classes (class 1 <50 x 10^6 sptz ml^{-1}, 2 = 50 to 100 x 10^6 sptz ml^{-1}, 3 = 100 to 150 x 10^6 sptz ml^{-1}, 4 = 150 to 200 x 10^6 sptz ml^{-1}, 5 = 200 to 250 x 10^6 sptz ml^{-1} and velocity (class 1 = 0.5, 2 = 1, 3 = 2, 4 = 3 e 5 = 4) of bovine frozen semen.

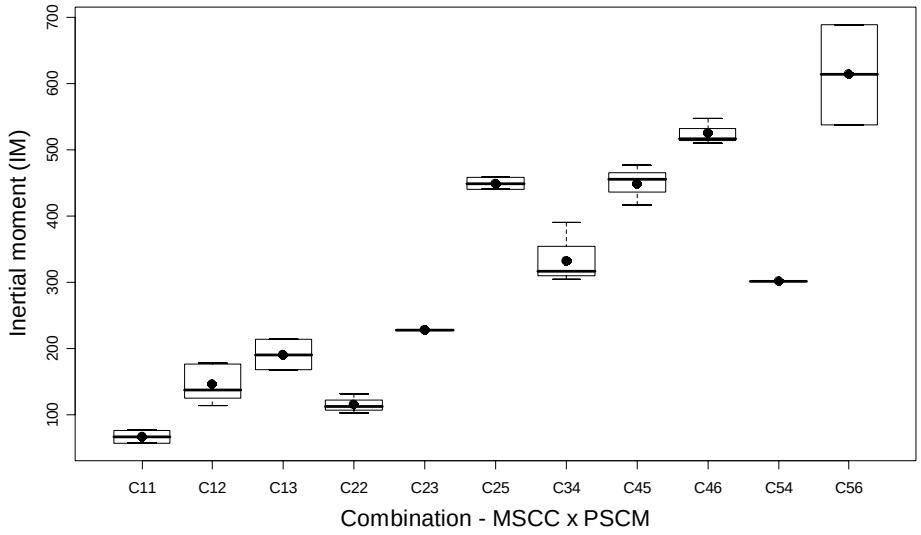

FIGURE 3- Box plot for inertial moment (IM) data values, according to the combinations of mobile sperm cell concentration (MSCC) classes (class 1 <50 x 10^6 sptz ml^{-1}, 2 = 50 to 100 x 10^6 sptz ml^{-1}, 3 = 100 to 150 x 10^6 sptz ml^{-1}, 4 = 150 to 200 x 10^6 sptz ml^{-1}, 5 = 200 to 250 x 10^6 sptz ml^{-1} and percent sperm cell motility (PSCM) (class 1 = 5%, 2 = 10%, 3 = 20%, 4 = 40%, 5 = 55%, 6 = 75%) of bovine frozen semen.

3. ORIGINAL PAPER II

Biospeckle Laser (BSL) Technique Evaluation of Frozen Bovine Semen Using Two Different Laser Wavelengths.

(Preparado de acordo com as normas da revista "Reproduction")

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3.1 Abstract

Laser incidence on biologically active materials generates interference images known as biospeckle. Processing of these images generates an index (inertial moment - IM), which quantifies the activity of the material. This study was aimed at testing two different sources of laser light (red, He-Ne with 632 nm, 10 mW, and green, Solid State with 543 nm, 3 mW) for evaluation of bovine frozen semen, comparing the IM, obtained by application of the biospeckle laser (BSL) technique, with motility parameters evaluated by light microscopy (LM). A hundred and twenty-three straws were thawed and evaluated microscopically as to velocity and motility. In the assay 1, each sample was lit by either kind of laser (red, n = 56; green, n = 20). In the second assay 46 samples were lit alternately by both laser light sources. An index grouping together velocity and motility named the velocity and motility index (VMI) was proposed as a single estimate to compare LM evaluation and the IM generated by BSL. A regression study was performed between VMI and IM. In the third assay illuminations were done of a same sample along the time, with both sources of laser, to assess

motility decreasing, and a temporal regression analysis IM and VMI have been done. Biospeckle data were captured by a camera and transformed into matrixes from which IM was calculated. Velocity (V), percent sperm cell motility (PSCM) and VMI evaluated by microscopy was compared with IM. In the first assay, a positive correlation ($p < 0.01$) was found between IM and V ($r = 0.594$; $r = 0.734$) and IM and PSCM ($r = 0.665$; $r = 0.684$) for the samples illuminated by red and green laser, respectively. In the second assay, correlation coefficients between IM and V ($r = 0.801$; $r = 0.590$), IM and PSCM ($r = 0.826$; $r = 0.613$) and IM and VMI ($r = 0.840$; $r = 0.618$) for red and green laser, respectively were found ($p < 0.01$). In the third assay, a quadratic behavior of IM values over the time for the two laser sources was observed (red, $R^2 = 0.963$ and green, $R^2 = 0.984$). VMI presented a linear adjustment as related to time for both laser sources (red, $R^2 = 0.849$ and green, $R^2 = 0.961$). Results indicate that laser can be an useful tool for some features of bovine frozen semen evaluation and red and green lasers are sensitive in detecting the semen biological activity.

3.2 Introduction

Semen quality determinants include sperm concentration, morphology and motility. Guidelines used in Veterinary Medicine to semen analysis suggest that a bull, to be considered a potential breeder, should have at least 60% (Barth 2000) to 70% motile sperm (Henry & Neves 1998). Fertility and sperm motility were positively correlated in cattle (Neville *et al.* 1988) and in the human species (Marnet *et al.* 2000). Iguer-Ouada & Verstegen (2001) argued that percent motility sperm evaluated by conventional light microscopy has been a subjective component of semen analysis mentioning variances ranging from 30-60%, when distinct technicians evaluated the same ejaculate. In addition to the uncertainty of the routine methods, poor correlations between fertility and spermatozoa motility were described in some species (Nie *et al.* 2002).

Many efforts have been done to increase the objectivity of the routine methodologies to analyze the attributes of the semen samples, and one line that presents some works is the optical metrology. One account of optical evaluation can be reported to the approach presented by Ross *et al.* (1983) that used the laser illumination of the samples and the Doppler effect evaluation and correlation with semen motility.

Other methodology using optical approach was presented by Briers (1975) and Asakura & Takai (1981) applied to blood flow, been known as dynamic speckle or biospeckle laser techniques. The use of biospeckle laser as a tool to evaluate the flux was defended by Zhao *et al.* (1997), presenting better results related to the random movements in other botanical applications. The adoption of the dynamic speckle to measure some features in semen samples could be considered as an alternative approach that should use the facility of the methodology in flux evaluation, to measure biological activity of samples.

The incidence of laser light upon active materials, such as semen, generates successive interference patterns known as biospeckle. The patterns observed present an interference figure composed by grains with distinct bright levels, presenting a boiling effect in accordance with the material activity. The correlation between the intensity of the particle movements, responsible to the scattered light, and the rate of pattern change is the aim of the works to use that phenomenon as a source of information.

The procedures developed and under development to analyze those sort of images, speckle patterns under changing, have been based mainly in statistical methods, some presenting image outputs, others numerical values, and with a range of applications accounted by Briers (1975), Asakura & Takai (1981), Oulamara *et al.* (1989), Xu *et al.* (1995), Arizaga *et al.* (1999), Pajuelo *et al.* (2003), Rabelo *et al.* (2005), Braga *et al.* (2003; 2005; 2007).

The earliest works on spermatozoa (fish and rabbit sperm) showed that laser light scattering was strongly influenced by the motility of the cells, indicating its value as an assay of motility. First models (point scatterers) assumed that spermatozoa behave as point particles moving with constant velocity, however these models showed to be inadequate, because sperm cells, particularly from ram and bull, are large cells and far from spherical form. Harvey & Woolford (1980) have found that the interpretation of correlation functions on light scattered from bovine motile sperm requires a model which considers the size, shape, and unusual swimming trajectories of these cells. In addition, it was found that the spermatozoa exhibited a strong orientation effect, and the light scattered intensity was dominated by those cells swimming in a narrow angle which were almost perpendicular to the scattering vector.

Further studies (Woolford & Harvey 1982) have pointed to the importance of immotile cells presence in the ejaculate, interactions or collisions between motile and immotile cells, the distribution of spermatozoa within the scattering cell, spatial orientation effects and the shape of the bull spermatozoon to understand the spectrum or autocorrelation function of the scattered light by semen.

The adoption of the biospeckle laser technique was presented by Carvalho *et al.* (2005) in frozen bovine semen, Nascimento (2005) in ram semen and Braga *et al.* (2007) in horse semen both using samples *in natura,* showing positive correlation between the biospeckle laser activity values and the motility of the semen.

This paper evaluated the biospeckle technique as a tool to measure the quality of frozen semen samples under the view of motility parameters. The samples analyzed were from bovine reproducers under frozen process. The evaluation was conducted using two distinct wavelengths and the sample

behavior during time motility decaying, and the proposition of an index to join the velocity and motility attributes.

3.3 Materials and Methods

3.3.1 Semen samples

The semen straws were from a commercial company, American Breeders Service – Pecplan (ABS-PECPLAN - Uberaba**,** MG**,** Brazil**),** in the same conditions that they use to be sold. Semen was diluted in citrate-yolk extender, envased in 0.5 ml straws and frozen according routine procedures. The sperm cell concentration used to all experiments was 10 millions of viable cells, and total concentration was 30 to 35 x 10^6 sperm cells per straw. The samples were illuminated by two laser sets with distinct wavelengths and intensities in experiment 1, though with the same intensity upon samples in experiments 2 and 3.

3.3.2 Experiment I

The 76 samples illuminated by the two laser sets with different intensities (Helium-Neonium, red, 632 nm e 10 mW and Solid State, green, 632 nm e 3 mW) were analyzed by the biospeckle routine analysis, with the IM information as the result. The IM values were compared to routine semen evaluation using two evaluators, both trained in the same laboratory, which analyzed velocity (V) and percent sperm cell motility (PSCM) by LM.

The red (56 samples) and green (20 samples) illumination were conducted in distinct period. The straws were thawed at 37 ^oC for 30 seconds and 10 µl were evaluated microscopically to measure V (1 a 5) and PSCM (%) in slides with cover slips (15 x 15 mm), warmed in a hot platinum device (Henry & Neves 1998).

Each sample was lit by either kind of laser (red, n = 56; green, n = 20), after a 30 seconds period of stabilization before biospeckle readings.

3.3.3 Experiment II

The 46 samples were illuminated by red and green lasers alternately, respectively, using the same set up and without move the sample. The results accounted by IM were compared to V, PSCM and the velocity and motility index (VMI) proposed in this work. The new index was composed in accordance with the Eq. 1.

$$VMI = ½ (20V + PSCM) \quad \text{(Eq. 1)}$$

Velocity and motility index was proposed as a form of direct comparison of a single measure evaluated by light microscopy and the IM generated by BSL. The samples passed by the same period of stabilization accounted in the first experiment.

According to Eq. 1, the same relating value was given to V and PSCM because up to this experiment it was unknown if the influence of each parameter in BSL analysis was equal or not.

3.3.4 Experiment III

Illumination of a sample in time alternately with read and green laser in the same intensity were conducted, and the evaluations provided by IM and VMI compared. Eight illuminations were performed at room temperature within intervals of two minutes (0, 2, 4, 6, 8, 10, 12 and 14 minutes after thawing). To each BSL results obtained, another light microscopy evaluation was done.

3.3.5 Experimental setup

Experimental equipment consists of low power Helium-Neonium (He-Ne) and Solid State laser (10 mW and 3 mW of power, 632 and 543 nm of wavelengths, respectively), a beam splitter, a mirror, a colour digital charge-coupled detector device (CCD) and a computer with an image processor. A density filter was also used to warranty the same intensity of light in the sample to the both laser sets in the experiments 2 and 3. The 512 acquired images from the 640 x 480 pixels CCD were acquired in a rate of 0.08 seconds, and processed forming the STS patterns of 512 x 512 pixels, that are the data base adopted in the biospeckle image analysis and to get the IM, Arizaga *et al.* (1999). Figure 1 presents the configuration of the set up used, with the optical and image processing apparatus.

3.3.6 Statistical analysis

In the first experiment, the IM values, for the combinations found among the classes of V and PSCM investigated for the two sources of laser (red and green) were evaluated by means of an exploratory analysis; involving the calculation of the means and standard deviation.

The correlation coefficient between the variables V and PSCM, ascribed by two raters, and IM, were obtained and tested by the Spearman correlation test, with a nominal significance level of 5%. For all the statistic analyses, the statistic software R® version 2.5.0 (R 2007) was utilized.

In the second experiment, the values of IM obtained by red and green laser, for the combinations found between V, PSCM and VMI, were evaluated by means of an exploratory analysis, involving calculation of the means and standard deviations.

The correlation coefficient between the variables V, PSCM, VMI and IM evaluated under a source of red and green laser were obtained and tested by the Spearman correlation test with a nominal level of 5%.

The adjustment of a simple linear regression equation performed for the IM as related to the VMI for the sources of red and green laser. For all the statistic analyses, the statistical software Sisvar® version 4.0 (Ferreira 2000) and R® version 2.5.0 (R 2007) were utilized.

The third experiment analyzed the values of the IM and VMI, obtained by different sources of laser (green and red), adjusted simple linear regression equations were adjusted as related to the analyzed times, utilizing the statistic software R® version 2.5.0 (R 2007).

3.4 Results

3.4.1 Experiment I

The results from red laser illumination of the 56 samples and from green laser illumination of the 20 samples can be expressed by the Tables 1 and 2 that present the Spearman coefficients relating the percent sperm cell motility and the velocity for two evaluators with the inertial moment. The Tables 3 and 4 classified the features analyzed in groups of inertial moment ranges.

3.4.2 Experiment II

Table 5 shows that the index can represent the both features, once its value is in the same order of V and PSCM, been possible to summarize it in groups as presented in Table 6. That classification in groups allowed a positive correlation of the IM values with some defined ranges of VMI. Figure 2 presents fitted curves to IM versus VMI values under the red and green lasers, though the fitted curves expressed linear and quadratic behavior.

3.4.3 Experiment III

The behavior of the semen in time, under the two wavelengths, is presented in Figure 3 with the evolution of the IM in time fitted by a second order curve with R^2 about 0.98 to green and 0.96 to red laser. The Figure 4 presents the same evolution under the VMI that fitted the data by a first order curve, with R^2 of 0.96 to green and 0.84 to red laser.

3.5 Discussion

3.5.1 Experiment I

Variations between estimates made by the two evaluators were kept within 10% limits and so average values were used to subsequent analysis. Jequier & Ukombe (1983) observed variation coefficient of 44.3% among 26 technicians who estimated sperm cell concentration in a single semen sample using light microscopy. Similar results were obtained to motility and morphology evaluation. The small variation observed between the two technicians in this study was due to the standard training received in the laboratory that they belong to.

Inertial moment values ranged from 78 to 311 and 60 to 111 to red and green laser analyses, respectively. For both laser sources, increasing velocity and percent sperm cell motility values corresponded to progressively higher average IM values in the BSL analysis.

The mean values presented a clear tendency, but the individual relations expressed some stable states mainly in the velocity feature. Otherwise the percent sperm motility values presented reliable values in all configurations, which link with a strong relation between PSCM and IM, allowing the conclusion that the laser technique is better to evaluate activity related to the movement.

The results allowed the conclusion that the inertial moment presented coherent values to V and PSCM to both evaluators, under the two sources of light.

3.5.2 Experiment II

The regression curve between inertial moment and the VMI index showed that 83% of variation occurring in the IM values could be explained by variations that occur in the VMI index. The quadratic curve represents the green laser, and despite the adjusted same intensity in both lasers, the second order evolution can be explained by the lower wavelength of the green light (543 nm vs 632nm), that could pass through the sample and return with more light in a higher intensity with relation to the red laser. This results the second order behavior during the IM evaluation.

The results presented that the index proposed in this work is a reliable tool to compare at the same time V and PSCM with IM. The parametric characteristic of it can be pointed as one positive factor.

3.5.3 Experiment III

The off-set between the evolution of red and green laser curve over a sample, in the third experiment, reinforce the hypothesis about the ability of the lower wavelength to pass through the illuminated material, bringing more light from the background, and then increasing the IM values. The comparison between the curves in Figures 3 and 4 can be interpreted by the human tendency to force a continue decrease in the result of VMI (Figure 4), when in the no subjective results of IM the curves present an expected tendency of a physical phenomena. Besides, the BSL analysis is not subjective and inertial moment values are more precise to assess the behavior of a biological phenomenon.

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3.7 Tables

TABLE 1 – Spearman correlation coefficients estimate with their respective significances for all the variables velocity (V) and percent sperm cell motility (PSCM) ascribed by two raters (A1 and A2) and for their means (Me), and Inertial Moment (IM) on the basis of 56 samples of bovine frozen semen analyzed under red laser.

	V A1	*PSCM A1*	*V A2*	*PSCM A2*	*IM*
V A1	1.0000	0.8767 (p<0.0001)	0.8362 (p<0.0001)	----	0.5906 (p<0.0001)
V A2	0.8362 (p<0.0001)	----	1.0000	0.8631 (p<0.0001)	0.5436 (p<0.0001)
V Me	----	----	----	----	0.5945 (p=0.0002)
PSCM A1	0.8767 (p<0.0001)	1.0000	----	0.9228 (p<0.0001)	0.6747 (p<0.0001)
PSCM A2	-----	0.9228 (p<0.0001)	0.8631 (p<0.0001)	1.0000	0.6469 (p<0.0001)
PSCM Me	----	----	----	----	0.6651 (p<0.0001)

TABLE 2 – Spearman correlation coefficients estimate with their respective significances for all the variables velocity (V) and percent sperm motility (PSCM) ascribed by two raters (A1 and A2) and for their means (Me), and Inertial Moment (IM) on the basis of 20 samples of bovine frozen semen analyzed under green laser.

	V A1	*PSCM A1*	*V A2*	*PSCM A2*	*MI*
V A1	1.0000	0.7679 (p<0.0001)	0.8709 (p<0.0001)	----	0.6811 (p=0.0009)
V A2	0.8709 (p<0.0001)	----	1.0000	0.8461 (p<0.0001)	0.7999 (p<0.0001)
V Me	----	----	----	----	0.7342 (p=0.0002)
PSCM A1	0.7679 (p<0.0001)	1.0000	----	0.9378 (p<0.0001)	0.6563 (p=0.0017)
PSCM A2	-----	0.9378 (p<0.0001)	0.8461 (p<0.0001)	1.0000	0.7579 (p=0.0001)
PSCM Me	----	----	----	----	0.6840 (p=0.0009)

TABLE 3 – Mean values (standard deviation) of the Inertial Moment (IM) related to the classes of velocity and percent sperm motility for bovine frozen semen obtained with the red laser.

		Percent sperm cell motility classes[*]			*Mean*
		1	2	3	
Velocity classes[*]	1	101.79 (13.06)	145.16	----	106.13 (18.43)
	2	100.19 (15.77)	145.75 (37.40)	172.75	135.40 (38.51)
	3	----	158.96 (41.44)	180.21 (48.49)	176.79 (47.45)
Mean		101.29 (13.28)	149.84 (36.57)	179.94 (47.57)	

*Classes of percent sperm motility: 1 (≤30); 2 (30 a 50); 3 (>50). Classes of velocity: 1 (≤2); 2 (2 a 3); 3 (>3).

TABLE 4 – Mean values (standard deviation) of the Inertial Moment (IM) related to the classes of velocity and percent sperm motility for bovine frozen semen obtained with the green laser.

		Percent sperm cell motility classes[*]			*Mean*
		1	2	3	
Velocity classes[*]	1	64.96 (6.04)	65.82	----	65.13 (5.25)
	2	75.58	66.37 (3.95)	70.50	68.59 (4.88)
	3	----	80.85 (5.62)	87.74 (16.02)	85.44 (13.43)
Mean		67.08 (7.07)	71.73 (8.53)	85.28 (16.02)	

*Classes of percent sperm motility: 1 (≤30); 2 (30 a 50); 3 (>50). Classes of velocity: 1 (≤2); 2 (2 a 3); 3 (>3).

TABLE 5 – Spearman correlation coefficients estimate with their respective significances for all the variables velocity (V), percent sperm motility (PSCM), inertial moment (IM) and velocity and motility index (VMI) on the basis of 46 samples of bovine frozen semen analyzed under red and green lasers light.

	V	*PSCM*	*VMI*	*IM Red.*	*IM Green*
V	1.0000	0.9506 (p<0.0001)	----	0.8019 (p<0.0001)	0.5906 (p<0.0001)
PSCM	0.9506 (p<0.0001)	1.0000	----	0.8267 (p<0.0001)	0.6132 (p<0.0001)
VMI	----	----	1.0000	0.8403 (p<0.0001)	0.6186 (p<0.0001)
IM Red	0.8019 (p<0.0001)	0.8267 (p<0.0001)	0.8403 (p<0.0001)	1.0000	----
IM Green	0.5906 (p<0.0001)	0.6132 (p<0.0001)	0.6186 (p<0.0001)	----	1.0000

TABLE 6 – Mean values (standard deviation) of the Inertial Moment (IM) obtained with the green and red lasers related to the velocity and motility index (VMI) for bovine semen.

Laser light	**VMI classes*		
	1	2	3
Red	130.19 (31.26)	205.94 (69.36)	402.61 (261.25)
Green	187.75 (58.24)	248.29 (130.9)	545.69 (362.15)

*Classes of VMI: 1 (≤50); 2 (50 a 70); 3 (>70).

3.8 Figures

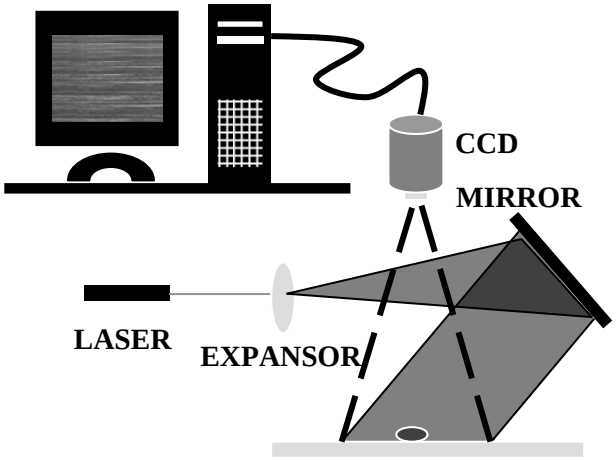


FIGURE 1: Equipment set-up

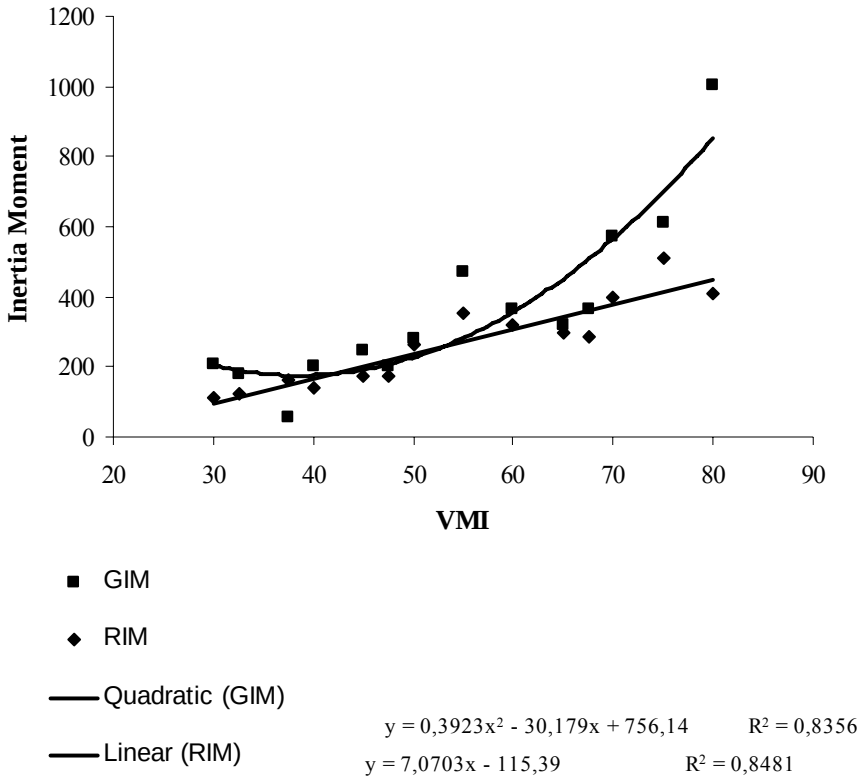


FIGURE 2 – Mean values of Inertia Moment (IM) obtained by the types of red (RIM) and green (GIM) laser related to velocity and motility index (VMI).

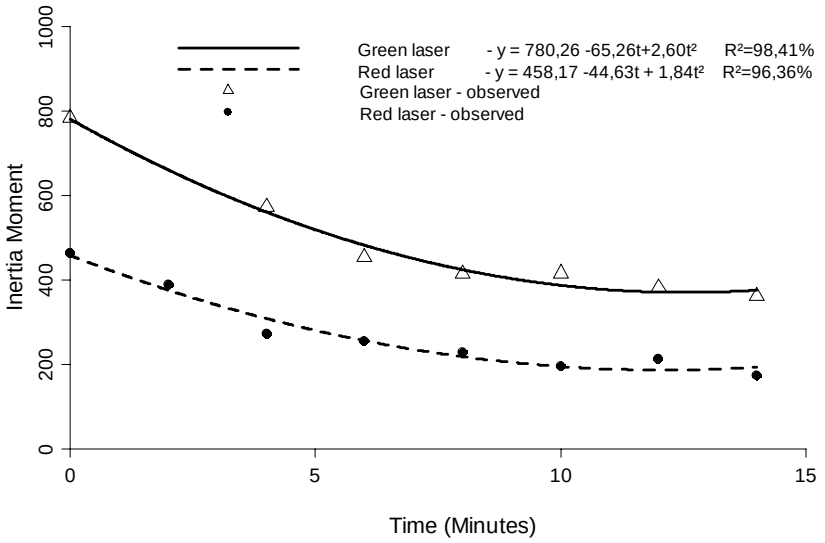


FIGURE 3 – Inertial moment (IM) values obtained by red and green lasers light related to the time.

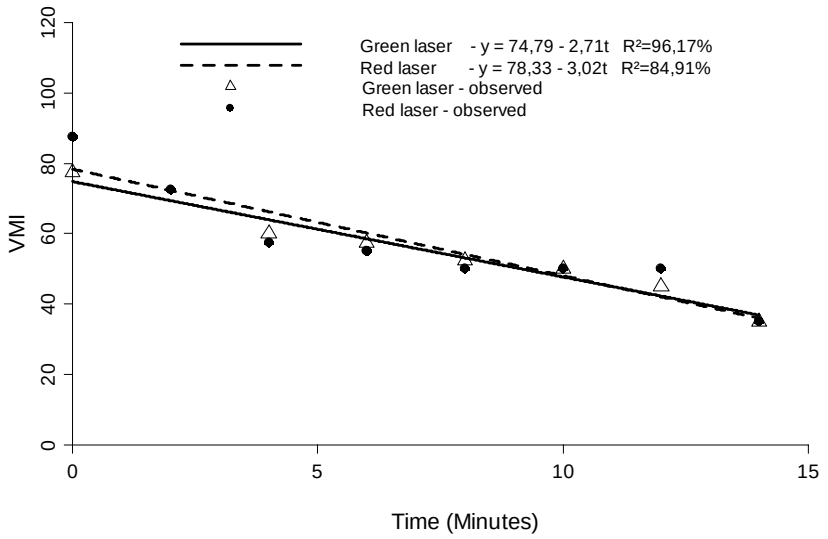


FIGURE 4 – Velocity and motility index (VMI) values obtained by microscopy evaluation for samples by red and green lasers light related to the time.

ANEXO

Simulação do cálculo do momento de inércia (MI)

A seqüência abaixo faz uma simulação da análise realizada pelo BSL, após a iluminação de um material biológico por luz laser e a aquisição das imagens do *biopeckle*, ilustrando a formação do STS (1º passo), MOC (2º passo), e cálculo do MI (3º passo), considerando-se apenas 6 tons na escala de cinzas, como mostra a figura 3.

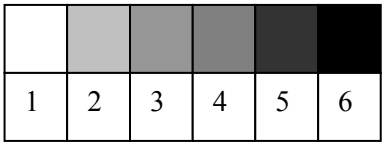
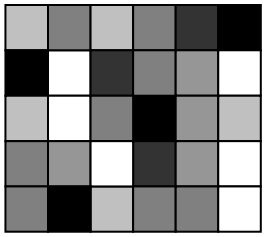


Figure 3 – Escala de cores em tons de cinza de 1 a 6.

1º Passo: Formação do *Spatial Temporal Speckle* (STS)



STS em tons de cinza

2	3	2	4	5	6
6	1	5	4	3	1
2	1	4	6	3	2
4	3	1	6	3	1
4	6	2	4	3	1

STS em escala numérica

2º Passo: Formação da matriz de ocorrências (MOC)

	1	2	3	4	5	6
1				1	1	1
2	1		1	2		
3	4	2				
4			3		1	2
5				1		1
6	1	1	2			

Matriz de ocorrências (MOC)

3º Passo: Cálculo do momento de inércia (MI)

$$\mathbf{MI = \Sigma\, N_{ij}\,(i-j)^2}$$

$$\mathbf{MI} = 0 + 0 + 0 + 1(1-4)^2 + 1(1-5)^2 + 1(1-6)^2 + 1(2-1)^2 + 0 + 1(2-3)^2 + 2(2-4)^2 + 0 + 0 + 4(3-1)^2 + 2(3-2)^2 + 0 + 0 + 0 + 0 + 0 + 0 + 3(4-3)^2 + 0 + 1(4-5)^2 + 2(4-6)^2 + 0 + 0 + 0 + 1(5-4)^2 + 0 + 1(5-6)^2 + 1(6-1)^2 + 1\,(6-2)^2 + 2(6-3) + 0 + 0 + 0$$

MI = 140