PE1.06 Fibre typing on bovine muscles sections using image analysis 79.00

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***Abstract*—Muscle fibre characteristics are involved in meat quality. The aim of this study is to propose a helpful method to evaluate the percentage of different fibre types using image analysis. Immuno-histological methods are practiced: cell outlines are identified by laminin antibody and cell type by their corresponding specific anti-myosin heavy chain. Different steps of image processing are developed. Fibre type percentages of two muscles studied (*Longissimus thoracis* and *Semi tendinosus*) in the "Blonde Aquitaine" breed are in agreement with results of literature. An accurate delineation of MyHC-based fibre types was obtained; automatic image analysis allowed a significant save in time. Hybrid fibres (containing simultaneously 2 types of MyHC) are easily characterized.**

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***Index Terms*— automatic quantification, bovine, fibre typing, image analysis, myosin heavy chain**

I. Introduction

Muscle fibre characteristics are involved in meat quality and play a key role in bovine meat tenderness. Fibres may be classified according contractile and/or metabolic properties. Among the numerous histochemical classifications, the most widely accepted is based on differences in the acid and alkaline stability of the myofibrillar ATPase reaction. This method allows separation between acid-resistant or slow fibres I and alkali-resistant or fast fibres II, these latter being distinguished as IIA and IIB fibres [1]. More recently, the use of monoclonal antibodies against myosin heavy chain (MyHC) allowed a more precise classification. The differential resistance to acid or alkali pre-incubation depends in trunk and limb muscle of adult cattle, on the presence of specific isoforms of myosin heavy chains (I, IIa, IIx). The conventional bovine IIB fibre based on mATPase has been identified to correspond with MyHC–IIx [4,10]. Important interest on the use of monoclonal antibodies is the delineation of hybrid fibres (I-IIA and IIA-IIX) (For review see [5]).

All these classifications can be seen in the visual comparison of different serial transverse muscle sections. This approach is very laborious and time consuming. Use of automated image analysis was then proposed in relation with the development of computer software. Thus, Lefaucheur, Buche, Ecolan & Lemoing (1992) had proposed a computer-aided method for muscle fibre type identification, based on ATPase staining [3]. Afterwards Quiroz et al developed various studies on fibre typing from different species [8]. Using immunohistochemical methods to characterize different MyHC, they presented a coordinated expression of MyHC, metabolic enzymes and morphological features. Optical density value from image analysis on cells was corroborated with fibre typing by visual examination.

It is well known that muscles with a large proportion of fast glycolytic fibres have a higher speed of ageing [11]. In order to evaluate influence of percentage of different fibre type on meat quality and even to study at the cellular level, the effect of different technological treatments, it was of interest to develop an automatic method for fibre typing, in continuity with previous studies.

In different scientific papers, "Image analysis" terms are often mentioned about histological studies. Few explanations or details are generally given on the methods used to do the treatments and to characterize fibres, positive reaction with specific staining, etc… The aim of this research is to propose a useful and precise automated method to evaluate the percentage of different fibre types. Explanation of different steps of image processing will be presented. Employing the developed method, two bovine muscles will be compared.

II. Materials and Methods

Three young bulls (16 months old) of the "Blonde Aquitaine" breed were slaughtered at the experimental INRA slaughter house. On the 1st day *post mortem,* two muscles: *Semi Tendinosus* (ST), and *Longissimus thoracis* (LT) were excised and samples (1x1x1 cm) were promptly frozen in isopentane chilled by liquid nitrogen (-160°c) and stored at -80°C. Serial transverse sections (10 µm thick) were obtained from each sample with a cryostat (Cryo-star HM 560 Microm international

GmbH, Germany) at -15°C, mounted on glass slides and stained using immunohistological methods.

Sections were preincubated in a blocking solution of stock goat serum (G 9023 Sigma), 10% in PBS. The cells outline were stained using a rabbit anti-laminin primary polyclonal antibody (L9393 Sigma) with a Goat anti-rabbit IgG Cyanine Cy3-labeled secondary antibody (111-165-008, Jackson).

For contractile fibre type determination, to identify slow and fast myosin heavy chains isoforms (MyHC) in the bovine muscle fibres, mouse monoclonal antibodies (mAbs) specific to MyHC isoforms were used : BA-D5 specific of MyHC-I and SC-71 for MyHC-II [9], were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany); S5 8H2 which reveals MyHC-I and MyHC IIx was purchased from AGRO-BIO (La Ferté Saint Aubin, France). It was obtained from hybridoma between SP2/O-antigen 14 myeloma and lymphocyte of mice immunised with a purified chicken and pig myosin mix. Hybridoma production was part of an INRA project for IIa or IIb MyHC antibody production in different species. In bovine muscle, this antibody is specific to I and IIx MyHC isoforms [6]. The myosin isoforms were revelated by an Alexa Fluor 488 labelled goat anti-mouse IgG secondary antibody (A11001, Invitrogen).

To reveal the antigenic expression in muscle fibres, the muscle cross-sections were incubated with both primary antibodies in a humidified box overnight at 4°C. After washing, primary antibodies binding was revealed by incubating 1 hour in the dark, at room temperature, with both labelled secondary antibodies (Alexa 488 anti-mouse IgG and CY3 anti-rabbit IgG). After new washes of the slides, they were cover slipped with Fluoromount (F4680, SIGMA, USA)

Histological sections were visualized under an Olympus motorized fluorescence microscope BX 61, using the 10x objective and the adequate band pass filter. High resolution greyscale images (1360 x 1024 pixels representing a 877 x 660 µm² field of view) were acquired with an Olympus digital camera DP 71 under Cell F software (Olympus Soft Imaging Solutions). Image processing was realized with a home-made visual basic program developed under the Visilog 6.7 Software (Noesis, France). Five fields were analysed on each serial cut.

III. RESULTS AND DISCUSSION

The colour image is the result of sequential dual-pass imaging with two greyscale images digitized with the camera, corresponding respectively to laminin revealed with CY3 and MyHC revealed with Alexa fluor 488. The microscope is equipped with a filter wheel to switch the excitation wavelength and a matching associated filter set. Then the red and green fluorescence are excited subsequently and two different monochrome images automatically acquired. The colour image is generated by combining the two monochromatic images each one being associated with its matching colour. Automated microscope allows the use a shutter driven by cell F menu which limits problem of photobleaching of the dye. Alexa Fluor 488 and CY3 are bright fluoprobes less sensible to photobleaching than traditional probes. For each fluorescent probe, acquisition done in grey levels allows a higher sensitivity and shorter acquisition time.

Different images are derived using the following antibodies:

BAD5 Mab (Fig. 1) - The produced image, on LT muscle, corresponds to a well defined characterization of the type I fibres. Separate adjustment of the acquisition time for each fluoprobe allows getting a contrasted image. Negative cells may not be type I, this eliminates for negative cells to be hybrid like I-IIA fibres.

SC71 Mab (Fig. 2) - Image obtained with SC71 Mab gives a complementary image of those obtained with BAD5. This result allows confirming the good selection of marked cells given with BAD5 Mab. This marking is not necessary in routine to evaluate the percentage of different types except for hybrid cells I-IIA which are unusual (not taken in account for this experiment). BAD5 and SC71 Mabs give regular and well reproduced results.

S5-8H2 Mab (Fig. 3) marks both MyHC I and MyHC IIx. The unmarked cells correspond to pure MyHC IIa. The marked cells correspond to MyHC I already selected with BAD5 and MyHC IIx cells which may be pure MyHC IIx or hybrid MyHC IIa-IIx. The S5-8H2 marked cells unmarked with BAD5 are corresponding to these last two categories.

The following steps of images analysis are practiced with Visilog 6.7 thanks to an algorithm and an easy to use graphical user interface developed in our Institute. Using the laminin component of the laminin-BAD5 marking, the greyscale image (Fig 4) is thresholded which allows after binarisation, segmenting of the laminin

network. Watershed lines are used to automatically close this network resulting in a good separation of objects corresponding to muscular cells. Random colour distinguish between each cell (Fig. 5). The incomplete cells touching the border are eliminated. Area and morphological index are calculated for each cell. At this level, it is possible to manually add, suppress fibres or correct bad segmentation. The result gives the total cells isolated for the fibre type discrimination. On each field, on average, around 120 cells are characterized which represents 600 fibres by cut.

The greyscale image of BAD5 MyHC marking is thresholded (Fig. 6) and compared to total cells image (Fig. 5) giving the cells positive for MHC I if at least 50% of their area is marked.

An equivalent treatment is applied after adding the S5-8H2 marking. First, it is necessary to align the laminin network of both laminin-BAD5 and laminin-S5-8H2 images to match the cells. The warp command is used to correct the geometric distortions between the serial histological sections. To this end vectors are manually drawn between matching points (red line on Fig. 7) of the two networks. A deformation model is computed from these registered points and it is applied to S5-8H2 marking. Positive cells with S5-8H2 marking are sum of IX, IIA-IIX hybrid fibres and pure IIX fibres. I fibres have already been characterized with BAD5 antibody. For IIA-IIX hybrid fibres, fluorescent signal is less intensive than for pure IIX fibres (Fig 8). A two level step of thresholding allow separation of these two categories. Intermediate level thresholding shows hybrid IIA-IIX fibres (Fig. 9), high level thresholding shows the pure IIX fibres which present high level of fluorescence (Fig. 10). By coupling together the total identified cells, respective BAD5 and S5 8H2 marking and geometric correction between laminin networks, we manage to identify fibre types of the cell in a quick, automatic and precise way. Fibres are separated between fibres I slow (red), fibres IIA (yellow), hybrid fibres IIA-IIX (green) and fibres IIX (blue) (Fig. 11). Morphological (cross-sectional area, length, width, perimeter, shape) features of individual myofibres are attainable if necessary.

We wanted to evaluate pure IIX fibres by use of BF-35 Mab. specific of I and IIA fibres. Negative fibres with BF-35 will have been identified as pure IIX fibres. Unfortunately, BF-35 Mab used by different researchers in many papers does not seem available yet. The clone cell line seems to have lost its antibody production ability as confirmed by DSMZ company.

The method applied to the two different muscles LT and ST on three animals give the following results (Table 1). This results are in agreement with previous works on the same muscles by using the same antibodies [7]. The use of immunohistochemistry based on MyHC antibodies has been already described as a good method for the classification of muscle fibre types. The limit of this technique was the analysis of several serial sections which was time consuming. So, the automated proposed method of quantification of fibre types by an automatic superposition of serial sections allows the application of this method routinely on large numbers of samples without limitations.

	Fibres I	**Fibres IIA**	**Hybrid fibres IIA-IIX**	**Fibres IIX**
LT Proportion (%) (n= 2098)	**36**	**16**	**14**	**34**
ST Proportion (%) (n= 1475)	**8**	**14**	**10**	**68**
LT mean area μm^2 (s.e.m)	**1484** (24)	**1743** (48)	**2512** (75)	**3628** (62)
ST mean area μm^2 (s.e.m)	**1717** (93)	**1488** (64)	**2775** (130)	**4263** (67)

IV. CONCLUSION

An accurate delineation of MyHC-based fibre types was obtained with the immunohistochemical method developed. Interest of our work is the use of automated image analysis which allows significant time saving. This ability permits the evaluation on higher numbers of cells, giving a best representation of fibre types, particularly on heterogeneous muscle. As the BF-35 cell line has lost its ability to produce MHC antibody, the complete characterisation of fibre type is not yet available. It would be of great importance to find antibody against MyHC IIa or like BF35 against I+IIa.

This method may be applied for all different species of animals and for humans, adjusting the different antibodies used.

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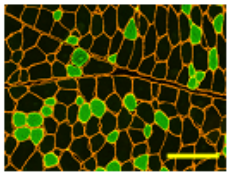


Fig.1 : Laminin and MyHC I (BAD5) dual marking. LT muscle

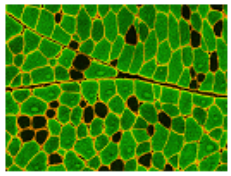


Fig.2 : Laminin and MyHC II (SC71) dual marking

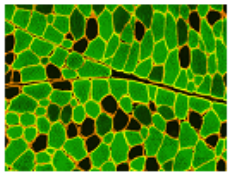


Fig.3 : Laminin and MyHC I-IIx (S5-8H2) dual marking

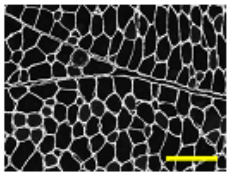


Fig.4: Greyscale laminin network marking

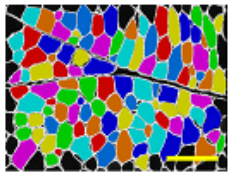


Fig.5: Total cells characterization

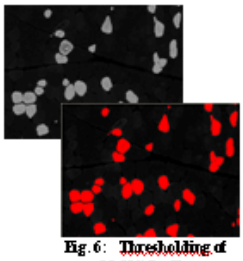
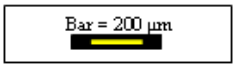


Fig.6: Thresholding of MyHC I cells



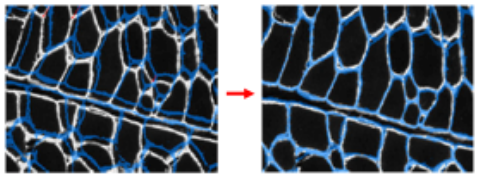


Fig.7: Warping step to overlay laminin network

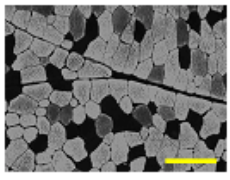


Fig.8: Greyscale MyHC I-IIx marking (S5-8H2)

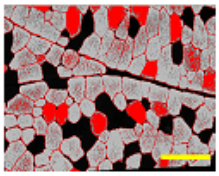


Fig.9: Intermediate thresholding of grey levels (hybrid fibres IIA-IIX)

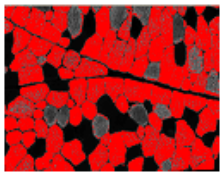


Fig.10: High thresholding of grey levels (fibres IIX)

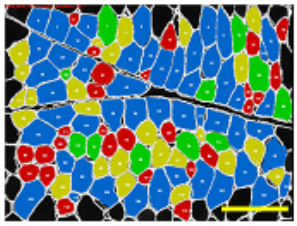


Fig.11: Final characterization of different fibre types