Alexandria Journal of Veterinary Sciences 2016, July 50 (1): 65-69
ISSN 1110-2047, www.alexjvs.com
DOI: 10.5455/ajvs.218223

Immunohistochemical localization of Haptoglobin in the Bovine Mammary Gland

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Key words:
Haptoglobin, Bovine, Mammary gland, Immunohistochemistry, mastitis

ABSTRACT

Haptoglobin (Hp), an acute phase protein has been recognized as an important indicator of intramammary infection that can be assessed in milk. In this study, we utilized immunohistochemical techniques on naturally infected and healthy bovine mammary glands in order to determine the source of Hp found in milk during bovine mastitis. Mammary glands sections were harvested from udder of two cows, one showing signs of clinical mastitis and the other healthy. Hematoxylin and eosin as well as immunohistochemistry (IHC) for anti-Hp staining were carried on these sections. It was revealed that the mammary epithelial cells around the alveolar ducts of the gland as well as with infiltrating neutrophils had dense staining for Hp in mastitic sections while in the healthy section there was minimal cytoplasmic positivity for Hp on the ductal epithelial cells. These observations support the conclusion that Hp arises locally from mammary epithelial cells and from circulation from migrating neutrophils during episodes of mastitis, and can therefore serve as a sensitive indicator of bovine mastitis.

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1. INTRODUCTION

Bovine mastitis is a leading cause of economic losses to the dairy industry worldwide (Samaha et al, 2012; Halasa et al., 2007) and a major hindrance to its proper management in dairy establishments is the inability to promptly and adequately diagnose the subclinical form of this condition. While common diagnostic parameters such as somatic cell counting (SCC), California mastitis test (CMT) as well as less commonly used measures such as electrical conductivity (EC), infra-red thermography (IRT), lactose, measurement of milk enzymes namely lactate dehydrogenase (LDH) and N-acetyl-β-D-glucosaminidase (NAGase) have been used, problems of lack of sensitivity and specificity of these various parameters have arisen thereby highlighting the need for more sensitive parameters in milk for recognition of subclinical mastitis (Pyörälä, 2003).

Recently, acute phase proteins (APP), a group of protein which are synthesized predominantly in the liver in response to cytokine stimulation following inflammatory stimulus, such as mammary associated serum amyloid A3 (MSAA3), Haptoglobin (Hp) and C-reactive protein (CRP) have shown promise as potential markers of bovine mastitis in milk (Thomas et al., 2015). These proteins were initially thought to arise in milk due to seepage from blood following breakdown of blood-milk barrier during mastitis (Eckersall et al., 2001), however, other studies have shown involvement of local synthesis of these APP in the mammary gland (Mcdonald et al., 2001; Eckersall et al., 2006; Hiss et al., 2004).

In the study of Hiss et al. (2004), a real time polymerase chain reaction (RT-PCR) (quantitative and qualitative) identified synthesis of Hp in the mammary gland and it was later confirmed by IHC analysis of mammary gland sections following experimental mastitis, that Hp was synthesized in portions of the mammary gland (Hiss et al., 2005), although in that study, a significant difference between control and infected glands in the IHC staining of Hp, was not found. Further studies by Lai *et al*. (Lai et al., 2009), using immunocytochemistry (ICC) along with RT-PCR, demonstrated that neutrophils and mammary

epithelial cells (MECs) were major sites of Hp synthesis. Local synthesis of these APP can further improve their ability to sensitively indicate changes in the mammary gland due to intramammary infections/inflammation (IMI).

Immunocytochemical localization (which focuses on the localization of antigens within the cells) and differential expression of the APP MSAA3 was carried out on bovine mammary gland after an experimentally induced subclinical *Staphylococus aureus* mastitis(Eckersall et al., 2006). In that study it was observed that M-SAA3 was located most in the secretory mammary epithelial cells (MECs), gland cistern, and, to a lesser extent, teat canal.

Another APP, α-acid glycoprotein (AGP), a lipocalin, was immunohistochemically identified in all areas of a section of normal bovine mammary gland and it has been suggested production occurs in the alveoli (Ceciliani et al., 2007).

In order to understand the potentials of Hp as a biomarker of mastitis in milk, this study was carried to utilize the IHC techniques to localize areas of Hp synthesis in bovine mammary gland undergoing naturally acquired mastitis as against previous studies using experimental models of mastitis, caused by a major mastitis causing pathogen; *Escherichia coli* (*E. coli*) and to observe possible differences in the IHC localization of Hp in mastitic and non-mastitic glands.

Objectives: To characterize the source of milk Hp, during natural case of *E. coli* caused mastitis by IHC localization of Hp in the bovine mammary gland.

2. MATERIALS AND METHODS

2.1 Reagents

General chemicals were obtained from Sigma-Aldrich, Poole, UK, except where otherwise stated. In all experiments, milli *Q* water was used.

2.2 Samples

Samples from the udder of two cows were obtained for this experiment. The cows were a 10 years old beef suckler cow with clinical mastitis and an age matched non-lactating beef suckler cow with no clinical evidence of mastitis. Sections were prepared by Veterinary Diagnostic Services of the University of Glasgow, United Kingdom.

2.3 Tissue Sectioning

Tissues were fixed in 10 % neutral buffered formalin and embedded in paraffin wax. They were then cut using a microtome to a thickness of 2.5 microns and affixed onto a slide. Slides were baked at 57°C for 1 hour (h) prior to staining.

2.4 H&E staining

Sections of both healthy and mastitic mammary glands were stained with the haematoxylin & eosin (H&E) stain according to the conventional method as described by Bancroft et al., 2013.

2.5 Immunohistochemical staining

The IHC procedure was carried out according to the method described in Wolfe et al., 2013, with slight modifications. Briefly, prior to staining, tissue slides were deparaffinised in xylene and then rehydrated through graded ethanol solution, then placed in tap water.

The IHC was carried out using an automated IHC system by Dako (Agilent technologies, UK). All procedures were carried out at room temperature. Tissue sections were placed on to a Dako autostainer and then rinsed with buffer (Tris buffer pH 7.5 + Tween) after which sections were blocked for 5 min with Dako REAL™ peroxidase blocking solution. Sections were buffer rinsed for 5 min and the primary antibody, rabbit antibovine Hp (*Life Diagnostics*, USA) which was diluted to an optimum of 1:800 (1.72 µg/ml) in Dako universal diluent, was applied for 30 min.

Subsequently two 5 min buffer washes were carried out and then sections were incubated with a secondary antibody (Dako Envision system HRP labelled polymer anti-rabbit IgG (Dako UK Ltd, Cambridgeshire, UK). Two 5 min buffer washes were performed and the substrate, diaminobenzidine (DAB) (Dako DAB, K5007) was applied for 10 min. After 3 rinses with tap water, sections were counterstained for 27 seconds using Gills haematoxylin prepared in-house and finally sections were washed in water, dehydrated, cleared and mounted in synthetic resin.

2.6 Image Capture

Images of slides were acquired and analysed using an Olympus™ BX51 microscope (Olympus Life Science, Hamburg Germany) and were processed using a Cell^D imaging software (Electro Optics, Cambridge, UK). The images were captured using an Olympus DP71 digital camera. Sections were examined at magnifications of x100 and x200.

3. RESULTS and DISCUSSION

Gross images of both mammary glands used for this study are shown in Figure 1.

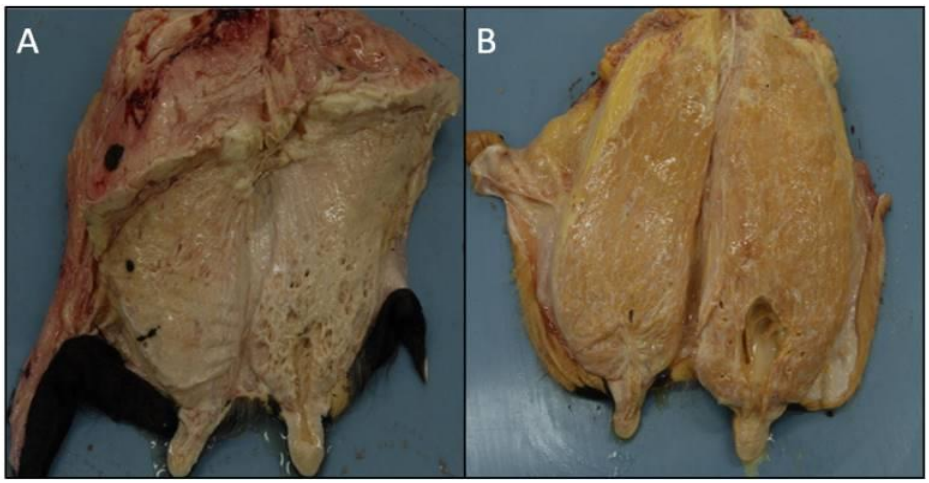

Fig. 1: Gross images of healthy (A) and mastitic (B) udders used for the study

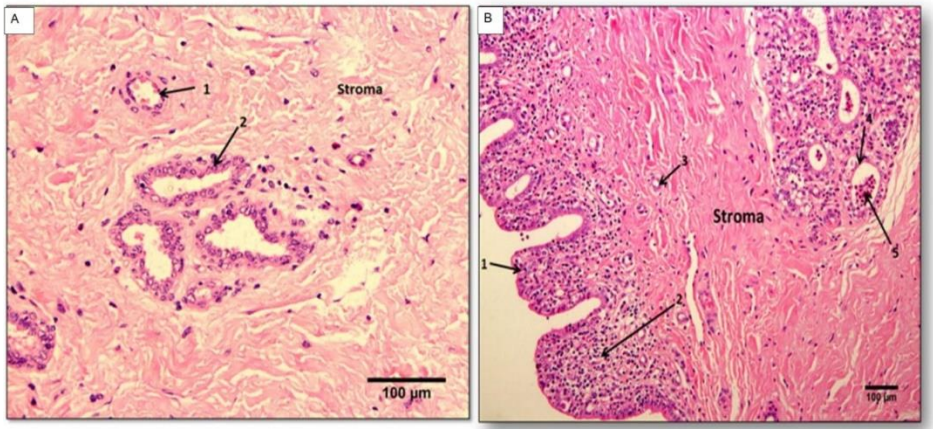

Fig. 2: A; Healthy (involuted) bovine mammary gland section, H&E, x200. Showing the normal mammary gland architecture, with occasional mononuclear cells infiltrating the periductular and perivascular stroma. Arrows indicate a blood vessel (1) and alveolar ducts (2). B; Mastitic bovine mammary gland section, H&E, x100. Arrows show duct epithelium (1), lamina propria (2), blood vessel (3), alveolar epithelium (4) and leuckocytes within the alveolar duct (5). Moderate numbers of neutrophils as well as lower numbers of lymphocytes and plasma cells, macrophages infiltrate the lamina propria and extend into the mucosal lining of the duct cistern and alveoli in the mastitic glands. Moderate numbers of neutrophils are seen within the alveolar lumens

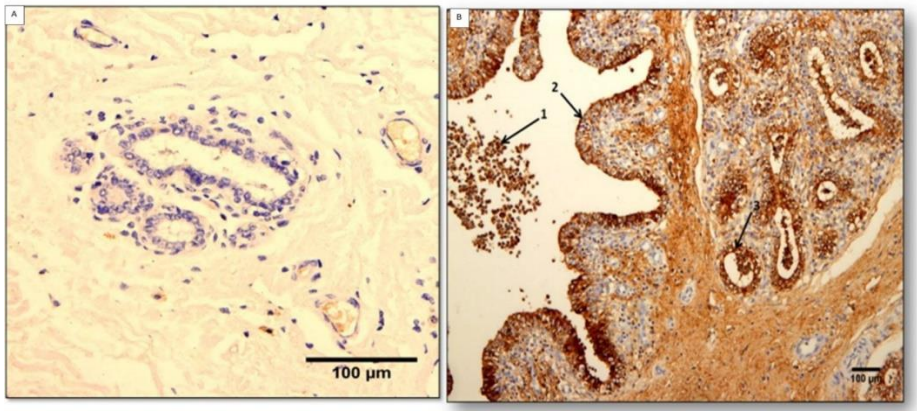

Fig. 3: A; Haptoglobin immunohistochemistry staining of a healthy (involuted) bovine mammary gland, x200: Very occasional ductal epithelial cells demonstrate minimal cytoplasmic positivity for Hp (brown staining). B; Haptoglobin IHC) staining of mastitic bovine mammary gland section, x100. Arrow showing intensely staining neutrophils in the duct lumen (1), duct epithelium (2), alveolar ducts with Hp stained leukocytes (3).

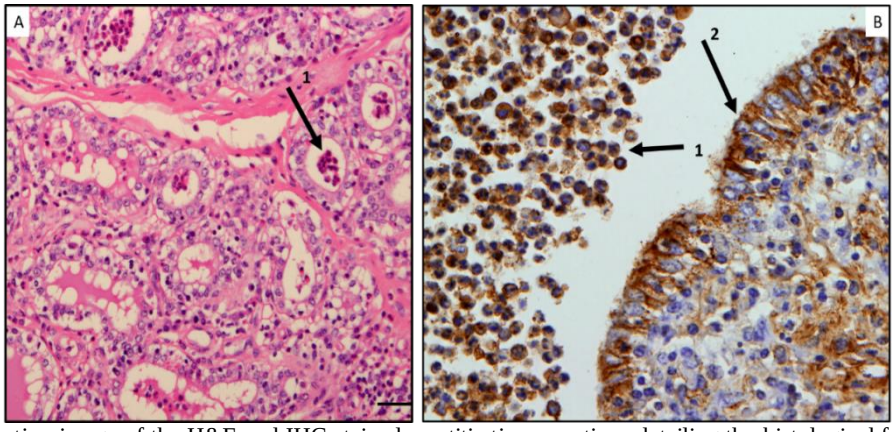

Fig. 4: Higher magnification image of the H&E and IHC stained mastitic tissue sections detailing the histological features described in Fig 2B and 3B above.

In this IHC study of normal and mastitic bovine mammary glands, it was found that there was marked staining for Hp within the infiltrating leukocytes and MECs during the natural form of mastitis. This agrees with previous reports of studies carried out in experimentally infected glands of the neutrophils and MECs as one of the major sources of milk Hp (Lai et al., 2009; Thielen et al., 2007) since it had already been established that Hp can be synthesized in other tissues apart from the liver (D'Armiento et al., 1997). However in addition to the alveolar epithelia cells, strong Hp staining was also observed for the ductal epithelial cells of the gland in this study.

Furthermore, because MECs of both alveoli and ducts of the gland were found to stain strongly for Hp, it can be inferred that the synthesis of Hp occurs in sites of frequent communication between the cells in the body and the exterior environment lending further support to its possible role in the innate immune response. Similar findings on IHC localization of Hp have been observed for porcine lungs (Hiss et al., 2008) and bovine female reproductive tract (Lavery et al., 2003, 2000), showing that the protein is synthesized on the cells with frequent communication with the external environment, another example of which are the mammary gland epithelial cells seen in the present study.

Only an IHC was carried out in this study, and histological scoring was carried out to identify neutrophils as the major infiltrating leucocytes, although an ICC would more clearly enhance the features the neutrophils. Moreover, it has been shown that neutrophils are the predominant type of leukocytes found in milk in acute mastitis, with macrophages occurring to a lesser extent. Hence neutrophils also correspond to the larger percentage (70%) of somatic cells often counted in milk as an indicator of on-going IMI (Hassan et al., 2016; Pilla et al., 2013; Lai et al., 2009; Pyörälä, 2003).

Hp has been shown to be stored in the granules within neutrophils, and undergoes exocytosis at sites of infection or injury (Theilgaard-Monch et al., 2006), therefore, the finding of dense staining for Hp in infiltrating leukocytes may also indicate influx of neutrophils to mastitic gland in order to release Hp for innate response. However there is no documented evidence of the storage of Hp within MECs, as no granules have been associated with these cells.

The local production of Hp as can be concluded from the results of this IHC localization, offers added advantage in the utilization of Hp as a mastitis indicator, as affected glands are expected to secrete more Hp into milk than unaffected glands from the same animal, thus conferring specificity.

In conclusion, IHC studies have been used to demonstrate the presence of Hp in infiltrating leukocytes as well as the MECs of mammary glands naturally affected by clinical *E. coli* mastitis, confirming their origin as one of the possible sources of milk Hp.

4. ACKNOWLEDGEMENTS

Funding of studentship by th Tertiary Education Trust Fund- Nigeria and Federal University of Agriculture, Abeokuta Nigeria is gratefully acknowledged. The authors also wish to thank the Veterinary Diagnostic Services unit- University of Glasgow for providing bovine mammary glands used in this study.

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