

## Qualitative detection of haptoglobin mRNA in bovine and human blood leukocytes and bovine milk somatic cells

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**ABSTRACT:** Local expression of haptoglobin (Hp) mRNA in tissue homogenates from different sites of the bovine mammary gland has recently been established. Besides alveolar and stromal tissue, blood cells are one potential cellular location of Hp expression. The aim of this study was to investigate whether bovine leukocytes might contribute to the Hp mRNA transcripts found in the mammary gland after their migration into this organ. For this purpose RT-PCR was carried out with total RNA extracted from leukocytes in blood and from somatic cells in milk of three dairy cows. In comparison, human leukocytes of four donors were examined. Both bovine blood and milk somatic cells showed distinct signals for Hp mRNA in all samples in contrast to human leukocytes where Hp mRNA was detected in only two out of four donors after reamplification. In conclusion, the presence of Hp mRNA in blood derived leukocytes indicates that these cells contribute to the Hp mRNA transcripts found in the bovine mammary gland. It remains to be clarified whether other cells within the mammary gland are also capable of Hp mRNA synthesis.

**Keywords:** haptoglobin mRNA; cattle; human; blood; milk

Acute phase proteins (APP) are known to play an important role within the response to infection, inflammation, injury or tumour growth (Heinrich et al., 1990). They are generated mainly by the liver leading to significant alterations of their blood concentrations during an acute phase reaction (APR). Haptoglobin (Hp) has been described as a major APP in cattle (Eckersall and Conner, 1988; Eckersall et al., 2001; Gronlund et al., 2003).

Several recent studies on mastitis in dairy cows have provided data on Hp concentrations not only in blood but also in milk to further elucidate its role in the animal's response to this disease (Eckersall et al., 2001; Gronlund et al., 2003, 2005; Pedersen et al., 2003; Hiss et al., 2004). In particular, a significant increase of Hp concentrations in milk was observed only hours after mastitis had been induced experimentally (Gronlund et al., 2003; Pedersen et

al., 2003; Hiss et al., 2004). Moreover, compared to blood Hp concentrations recorded simultaneously, the increase in milk occurred earlier (Hiss et al., 2004). These findings indicate that Hp might be synthesised locally in the mammary gland. Indeed, Hiss et al. (2004) showed Hp mRNA expression in tissue homogenates obtained from different sites of the bovine mammary gland, namely the teat, cisternal region and glandular parenchyma, and also in mammary biopsies obtained during experimentally induced mastitis. Immune cells from blood are potential candidates for Hp mRNA expression because they migrate into the udder at an increased rate after the onset of an infection in this organ (Mehrzad et al., 2004). We therefore aimed at investigating the presence of Hp mRNA in bovine leukocytes from blood and in somatic cells from milk. Human leukocytes were examined in com-

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parison because of existing, however contradicting information on Hp expression in the blood cells of this species. Wagner et al. (1996) found no Hp mRNA expression in the entire population of human peripheral blood leukocytes, while Yang et al. (2000) reported Hp mRNA in peripheral eosinophilic granulocytes.

## MATERIAL AND METHODS

### Blood cells

For leukocyte preparation, venous blood was collected into tubes preloaded with heparin (5 000 IU per l blood; Heparin-Natrium-5000-ratiopharm, Ratiopharm GmbH, Ulm, Germany) from three dairy cows (German Holstein) and four human donors all appearing healthy. Leukocytes were isolated by a 20 min centrifugation (4°C) at 1 100 g (bovine blood) or 700 g (human blood), respectively, and the subsequent use of the resulting buffy coat. Contaminating erythrocytes were eliminated by two consecutive hypotonic lyses for 20 s in water. After adding an equal volume of 2× PBS, the solution was centrifuged at 100 g for 8 min at 4°C. The pellets obtained were washed in PBS and stored at –80°C.

The viability of the isolated blood cells was assessed by Trypan Blue staining to be above 85%, and cells were differentiated in a smear stained by the Pappenheim method (Romeis, 1989).

### Milk somatic cells

Milk samples were obtained from another three dairy cows (German Holstein). After disposal of the

foremilk, milk was collected aseptically, mixed with an equal volume of PBS and centrifuged (1 000 g, 15 min, 4°C). The cell pellet was washed twice and stored at –80°C.

### RNA extraction

Total RNA was prepared from the blood and milk derived cells by the single step method (Chomczynski and Sacchi, 1987; Chomczynski and Mackey, 1995). After treatment with DNase (DNase I RNase free, Roche, Mannheim, Germany) total RNA was quantified by determining the optical density (OD) at 260 nm. RNA integrity was verified by the OD<sub>260</sub>/OD<sub>280</sub> absorption ratio >1.6 and by denaturing gel electrophoresis.

### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (1 µg) was reversely transcribed into cDNA with 50 pmol random hexamer primers (Invitrogen, Karlsruhe, Germany), 4 µl 5× reaction buffer, 500 µM dNTPs each, 20 U ribonuclease inhibitor and 200 U reverse transcriptase (all Fermentas, St. Leon-Rot, Germany) at 65°C for 5 min, 27°C for 10 min, 42°C for 60 min and 99°C for 1 min in a 20 µl volume. Three microlitres of this cDNA were used for the PCR together with 20 pmol of the respective primers (Invitrogen, see Table 1), 100 µM dNTPs each, 5 µl 10× reaction buffer with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgCl<sub>2</sub> and 1 U *Taq* DNA polymerase (all Fermentas) in a final volume of 50 µl. PCR conditions of the respective primers are summarised in Table 2. Amplified products (10 µl) alongside DNA marker (ΦX174

Table 1. Sequence of PCR primers used (F – forward; R – reverse), PCR product length and reference source

Gene	NIH GenBank accession No.	Product length (bp)	Primer sequence 5'-3'
Bovine Hp	AJ 271156	174	F = GTCTCCCAGCATAACCTCATCTC <sup>+</sup> R = AACCCACCTTCTCCACCTCTACAA <sup>+</sup>
Human Hp	NM 005143	338	F = CCTGAATGTGAAGCAGTATGT <sup>++</sup> R = TTCTGTTTGAGTTTGATGAGC <sup>++</sup>
β-actin	AY 141970	226	F = CGTGGGCCCGCCCTAGGCACCA <sup>+++</sup> R = GGGGGCCTCGGTCAGCAGCAC <sup>+++</sup>

<sup>+</sup>Hiss et al., 2004; <sup>++</sup>Chen et al., 1998; <sup>+++</sup>Fitzpatrick et al., 2002

Table 2. PCR temperature and time conditions

	Fragment						
	bovine Hp		human Hp		β-actin		
Denaturation	95°C	1 min	95°C	1 min	95°C	1 min	
Cycle	denaturation	94°C	40 s	94°C	40 s	94°C	30 s
	primer annealing	55°C	20 s	55°C	30 s	55°C	30 s
	extension	72°C	20 s	72°C	40 s	72°C	1 min
	Number of cycles	37		34		30	
Final extension	72°C	5 min	72°C	5 min	72°C	5 min	
Cooling	4°C	∞	4°C	∞	4°C	∞	

DNA/*Bsu*RI (*Hae*III), Fermentas) were visualised on a 2% agarose gel by ethidium bromide staining (FluorImager SI, Amersham Pharmacia Biotech, Uppsala, Sweden) and analysed by image analysis software (Peak Finder, Image QuaNT™, Version 4.1, Molecular Dynamics). Negative results by this standard PCR method were to be confirmed by PCR reamplification using 1 µl of the first course PCR products.

Species specific Hp primers were applied to the samples of human origin since use of bovine Hp primers did not yield any PCR products from human liver RNA.

### Controls

Liver RNA served as positive RT-PCR control. Bovine liver was sampled from a local abattoir and total RNA extracted as described above. Human liver Poly (A)<sup>+</sup> mRNA was kindly donated by Dr. Jens Gruber, RWTH Aachen University, Germany,

0.075 µg of which were employed in RT-PCR. Water was used as negative RT-PCR control. In order to control any residual genomic DNA contamination each RNA sample was also subjected to RT-PCR without the addition of reverse transcriptase. β-actin served as internal control gene. Since bovine β-actin primers were used (Fitzpatrick et al., 2002), specificity of the resulting PCR products from human RNA was established by sequencing.

### RESULTS AND DISCUSSION

Bovine Hp mRNA was expressed in both blood leukocytes and milk somatic cells (Figure 1). This is the first report on Hp gene expression in these bovine cell populations. In advancing our recent observation that bovine Hp mRNA is present in tissue homogenates from different parts of the mammary gland (Hiss et al., 2004), we could herein assign leukocytes as one cellular source of mammary Hp mRNA expression. Since milk somatic cells

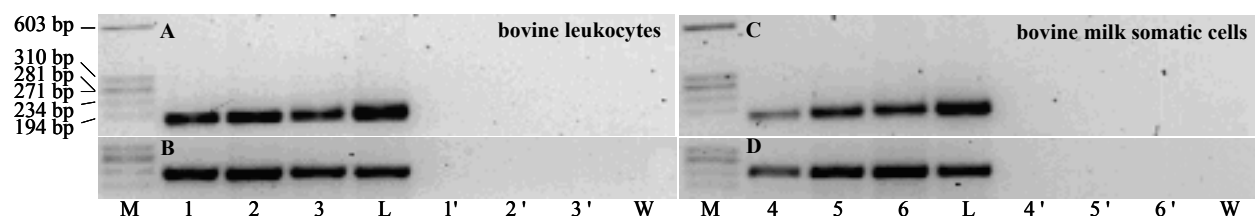


Figure 1. Qualitative RT-PCR detection of bovine Hp mRNA in bovine blood leukocytes (A) and milk somatic cells (C), parallel to the detection of β-actin mRNA as internal control in the same cells (B and D, respectively)

Lanes 1–3 and 4–6 contain DNA from RT-PCR of RNA from six individual cows. The same RNA samples underwent RT-PCR without the addition of reverse transcriptase (lanes 1'–3' and 4'–6'). M = marker, L = bovine liver (positive control), W = water control

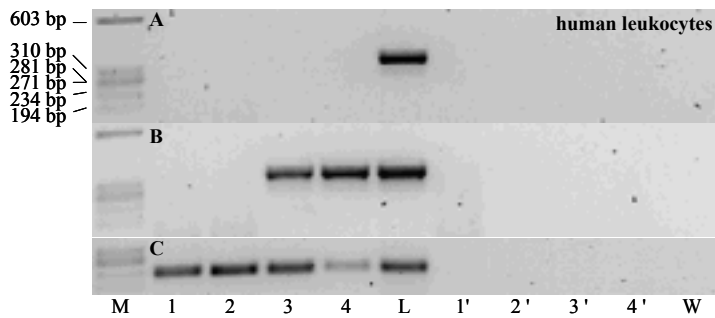


Figure 2. Qualitative detection of human Hp mRNA in human blood leukocytes after RT-PCR (A) and reamplification of 1  $\mu$ l of these first course RT-PCR products (B), parallel to the RT-PCR detection of  $\beta$ -actin mRNA as internal control in the same cells (C)

Lanes 1–4 contain PCR products of RNA from four individual test persons. The same RNA samples underwent RT-PCR and reamplification without the addition of reverse transcriptase (lanes 1'–4'). M = marker, L = human liver (positive control), W = water control

generally comprise about 90% blood-derived cells (Boutinaud and Jammes, 2002), the blood derived cells may contribute towards the Hp mRNA detected amongst the somatic cells in this study. However, our results could not clarify whether stromal or parenchymal cells of the mammary gland itself are capable of Hp mRNA synthesis. There is evidence that bovine mammary epithelial cells express other acute phase proteins such as lactoferrin (Molenaar et al., 1996), mammary-associated serum amyloid A 3 (McDonald et al., 2001) and mammary serum amyloid A 3 homologue (Molenaar et al., 2002).

In contrast to bovine leukocytes, no Hp mRNA was detected in human leukocytes by our standard RT-PCR method (Figure 2A), which is in agreement with results from the less sensitive Northern Blot analysis of human blood cells by Wagner et al. (1996). However, we found Hp specific transcripts after reamplification of the first course PCR products, but only in two out of four individuals (Figure 2B). Even in the cells of the fourth subject a distinct signal of Hp specific PCR products was observed after reamplification despite a reduced intensity of the band of the housekeeping gene  $\beta$ -actin. Yang et al. (2000) detected Hp mRNA in circulating eosinophils of normal healthy donors by in situ hybridization, but not in other blood cells. The differential cell count of our four test persons revealed 1% to 3% eosinophils of total leukocytes, all being in the normal range of 1% to 4% eosinophils in healthy individuals. The low abundance of this cell type might explain the absence of Hp transcripts by our standard RT-PCR protocol. In the examined white blood cells of the three cows the percentage of eosinophils was 2%, 4%, and 14% and, thus, within or close to the range of 2% to 10% eosinophils of healthy cattle. However, whether this cell type, like

in humans, contributes to Hp mRNA in leukocytes of bovines cannot be fully clarified by this study. Reamplification of PCR products is a tool widely used as part of the differential display method identifying and isolating genes differentially expressed (Wang et al., 1998).

All differential cell counts of the examined cows and humans showed normal or near-normal values for the respective species. Therefore, no infection was suspected to have induced an acute phase response, which could have partly explained the observed disparity between the two species in Hp mRNA expression. Basal extrahepatic Hp mRNA expression has also been reported in other cell types such as bovine oviduct cells or human keratinocytes (Lavery et al., 2004; Li et al., 2005).

Given our findings that Hp mRNA is expressed in circulating bovine leukocytes, these cells might consequently also be capable of synthesising the Hp protein and, thus, after their migration into the mammary gland might contribute towards the Hp protein in milk measured by other studies (Eckersall et al., 2001; Gronlund et al., 2003, 2005; Pedersen et al., 2003; Hiss et al., 2004;). In humans, granulocytes can store Hp and release the protein after their exposure to *Candida albicans* (Wagner et al., 1996) or to the cytokine TNF- $\alpha$  (Berkova et al., 1999). However, a well known difference between cattle and man exists concerning the extent of increase during APR. In cattle haptoglobin is considered to be the most prominent indicator of inflammation alongside serum amyloid A (Eckersall and Conner, 1988), whereas in man C-reactive protein is the most sensitive acute phase protein (Heinrich et al., 1990). Besides its well known haemoglobin binding properties, the effects of Hp have been described as modulating

the immune response based on trials with human and bovine sera showing inhibition of lymphocyte proliferation after exposure to Hp (Chase, 1972; Murata and Miyamoto, 1993). Similarly, specific binding of Hp to human neutrophils even inhibited their respiratory burst activity after stimulation by diverse agonists (Oh et al., 1990).

In summary, we could demonstrate that Hp mRNA is present in bovine peripheral blood leukocytes and somatic cells in milk. In humans, a signal for Hp mRNA could be detected only after reamplification in 50% of the examined individuals. Further research is required to determine whether also parenchymal and stromal cells of the bovine mammary gland produce Hp.

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