Glycoconjugate Journal 22, 109–118, 2005

Carbohydrate phenotyping of human and animal milk glycoproteins

Anki Gustafsson[1,4], Imre Kacskovics[3], Michael E. Breimer[2], Lennart Hammarström[4] and Jan Holgersson[4]

Departments of [1] Clinical Chemistry and [2] Surgery, Sahlgrenska University Hospital, Göteborg; [3] Department of Physiology and Biochemistry, Faculty of Veterinary Science, Szent István University, Budapest; [4] Division of Clinical Immunology, Karolinska Institutet, Karolinska University Hospital at Huddinge, Stockholm, Sweden

Breast-milk has a well-known anti-microbial effect, which is in part due to the many different carbohydrate structures expressed. This renders it a position as a potential therapeutic for treatment of infection by different pathogens, thus avoiding the drawbacks of many antibiotics. The plethora of carbohydrate epitopes in breast-milk is known to differ between species, with human milk expressing the most complex one. We have investigated the expression of protein-bound carbohydrate epitopes in milk from man, cow, goat, sheep, pig, horse, dromedary and rabbit. Proteins were separated by SDS-PAGE and the presence of carbohydrate epitopes on milk proteins were analysed by Western blotting using different lectins and carbohydrate-specific antibodies. We show that ABH, Lewis (Le)^x, sialyl-Le^x, Le^a, sialyl-Le^a and Le^b carbohydrate epitopes are expressed mainly on man, pig and horse milk proteins. The blood group precursor structure H type 1 is expressed in all species investigated, while only pig, dromedary and rabbit milk proteins carry H type 2 epitopes. These epitopes are receptors for *Helicobacter pylori* (Le^b and sialyl-Le^x), enteropathogenic (H type 1, Le^a and Le^x) and enterotoxic *Escherichia coli* (heat-stable toxin; H type 1 and 2), and *Campylobacter jejuni* (H type 2). Thus, milk from these animals or their genetically modified descendants could have a therapeutic effect by inhibiting pathogen colonization and infection.
Published in 2005.

Keywords: **milk proteins, carbohydrate epitopes, pathogens**

Abbreviations: **ER, endoplasmatic reticulum; MALT, mucosa-associated lymphoid-tissue; BSA, bovine serum albumin; Blood group antigens A: GalNAcα1-3[Fucα1-2]Galβ; B: Galα1-3[Fucα1-2]Galβ; H type 1: Fucα1-2Galβ1-3GlcNAc; H type 2: Fucα1-2Galβ1-4GlcNAc; Lewis a: Galβ1-3[Fucα1-4]GlcNAc; sialyl Lewis a: NeuAcα2,3Galβ1-3[Fucα1-4]GlcNAc; Lewis b: Fucα1-2Galβ1-3[Fucα1-4]GlcNAc; Lewis x: Galβ1-4[Fucα1-3]GlcNAc; sialyl Lewis x: NeuAcα2,3Galβ1-4[Fucα1-3]GlcNAc; pk: Galα1-4Galβ1-4Glc; P1: Galα1-4Galβ1-4GlcNAc.**

Introduction

Host cell adhesion of pathogens in humans and other species is a prerequisite for most infections and for many bacteria, viruses and bacterial toxins, binding is mediated by lectins that recognize and bind to different carbohydrate epitopes [1]. Today, the most common treatment for bacterial infections is the use of various antibiotics; a treatment that can be complicated by the occurrence of pathogenic strains resistant to commonly used antibiotics, as well as patient allergy to antibiotics. Studies in infants have shown that milk has a general anti-microbial effect that stems from the presence of antibodies specific for pathogens encountered by the mother, enzymes that may lyse micro-organisms, and proteins competing with bacteria for essential molecules such as iron [2]. In addition, milk contains free oligosaccharides and glycoconjugates such as glycoproteins and glycolipids [2–5] that are believed to confer protection of the infant against a variety of bacterial pathogens including *Helicobacter pylori* [6], *Streptococcus pneumoniae* and *Haemophilus influenzae* [7], enteropathogenic and S-fimbriated *Escherichia coli* [8,9], *Vibrio cholerae* [10], and their products, for instance Shiga and Shiga-like toxins [11,12]. The

To whom correspondence should be addressed: Anki Gustafsson, Division of Clinical Immunology, Karolinska Institutet, S-141 86 Stockholm, Sweden. Tel.: +46 8 585 877 85; Fax: +46 8 585 813 90; E-mail: anki.gustafsson@clinchem.gu.se

complexity of the milk carbohydrate repertoire differs between species [3], and it may be speculated that this repertoire in various species has evolved to protect the host against microbes pathogenic to that particular species.

In recent years, techniques used to generate transgenic animals have improved [13,14], and transgenic animals are now being used as bioreactors for production of recombinant proteins in their mammary gland [15,16]. The carbohydrate structures expressed in milk are produced and processed in the endoplasmic reticulum (ER) and the Golgi of mammary gland epithelial cells. Thus, glycosyltransferases transgenically expressed in the breast gland of different animals can be used to modify existing milk glycoconjugates for production of specific, humanized carbohydrate epitopes [17] resulting in milk that may have a therapeutic value in terms of prevention or treatment of microbial infections. This was recently shown in a model in which transgenic mice expressing the human α1,2-fucosyltransferase (FUT1), thus expressing H antigens in their milk, provided protection against *Campylobacter jejuni* infections in their suckling pups [18].

Although mice provide a good model for animal studies, production of larger volumes of milk with a potential therapeutic effect against pathogens, will necessarily have to be carried out in larger mammals. However, so far the expression of different carbohydrate epitopes in milk has been investigated in man and cow mainly, and a large part of those studies are confined to oligosaccharides and not to saccharides carried by proteins [3,19,20].

Protein-carbohydrate interactions are generally known to be of low affinity, and are characterized by weak chemical interactions. Nature's way of enhancing these interactions is multivalency, *i.e.* the simultaneous binding of multiple ligands on one biological entity, to multiple receptors on another [21]. Studies have also shown that a multivalent ligand displays a higher inhibitory capacity than its monovalent counterpart [22,23]. Consistently, the multiple expression of a specific carbohydrate epitope on a protein (or as a glycolipid in a micelle) may provide considerably higher protective properties towards a specific pathogen than the same carbohydrate epitope expressed as an oligosaccharide. Thus, phenotyping of protein-bound carbohydrate epitopes with a potential anti-adhesive effect on microbes is of particular relevance.

In order to identify animal species whose milk may have a therapeutic anti-microbial effect, or which could be genetically modified to produce a human spectrum of milk saccharides, we investigated the carbohydrate epitopes present on milk proteins from various species. We show that the ABH, Lewis $(Le)^x$, sialyl-Le^x, Le^a, sialyl-Le^a and Le^b carbohydrate epitopes were expressed mainly on man, pig and horse milk proteins. The blood group precursor structure H type 1 were, with varying intensity, expressed in all species investigated, while only pig, dromedary and rabbit milk proteins showed a clear staining with an anti H type 2 antibody.

Materials and methods

Chemicals

Bovine serum albumin (BSA) neoglycoproteins carrying defined carbohydrate epitopes (H type 1, Le^b, Le^x, sialyl-Le^x, 3'sialyl-LacNAc, and Galα1,3Gal) were purchased from Dextra (Reading, UK). Globotriose-APD-HSA were purchased from IsoSep (Tullinge, Sweden) and BSA was from Sigma Chemical Co (St Louis, MO, USA).

Lectins and antibodies

HRP-conjugated lectin *Griffonia simplicifolia* IB-4 (GSA IB-4), biotinylated lectins *Arachis hypogaea* (AHA) and *Ulex europaeus* I (UEA-I), and HRP-conjugated goat anti-mouse IgG [F(ab)'$_2$] antibody were obtained from Sigma. Biotinylated lectins *Erythrina cristagalli, Maackia amurensis* (MAA), *Sambucus nigra* (SNA), and *Vicia villosa* IB_4 (VVA) were purchased from Vector (Burlingame, CA, USA). Mouse anti-H type 1 (IgG_3, clone 17-206) and anti-Le^b (IgM, clone T218) antibodies were purchased from Signet (Dedham, MA, USA). Mouse anti-H type 2 antibody (IgM, clone 92FR-A2), mouse anti-A antibody (IgM, clone 81FR2.2) and mouse anti-B antibody (IgM, clone 3E7[1]) were obtained from Dako (Carpinteria, CA, USA). Mouse anti-sialyl-Le^x antibody (IgM, clone KM93), mouse anti-Le^x antibody (IgM, clone P12), mouse anti-sialyl-Le^a (IgG1, clone KM231), and mouse anti-Le^a antibody (IgG1, clone T174) were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Rat anti-CD77 (blood group p^k, IgM,k, clone 38-13) and HRP-conjugated goat anti-rat IgM antibody was purchased from Nordic BioSite (Täby, Sweden). Mouse anti-P1 antibody (IgM, clone 650) was purchased from Biotest (Dreieich, Germany). HRP-conjugated goat anti-mouse IgM antibody, HRP-conjugated goat anti-mouse IgG_3 antibody, and HRP-conjugated NeutrAvidin were purchased from Cappel (Durham, NC, USA), Serotec (Oxford, England), and Pierce (Rockford, IL, USA), respectively. For antibodies and lectins used, and their carbohydrate specificity, see Table 1.

Milk samples

Milk samples from man, cow, goat, sheep and pig were collected from two individuals of each species, while milk samples from horse, dromedary and rabbit was from one individual of each species. For cow, one colostrum sample was also collected. All samples—except the colostrum—were collected from mid-lactating animals that were in good health condition with no signs of clinical or subclinical mastitis.

Determination of the total protein concentration in milk sample

Milk samples were frozen immediately upon collection and stored at -20°C until further processed. The protein concentration in individual milk samples was determined using the

Table 1. Antibodies and lectins used in the western blot experiments with their known carbohydrate specificity

Name	*Clone*	*Carbohydrate specificity*
Anti H type 1	17-206	Fucα1-2Galβ1-3GlcNAc
Anti H type 2	92FR-A2	Fucα1-2Galβ1-4GlcNAc
Anti A	81FR2.2	GalNAcα1-3[Fucα1-2]Galβ
Anti B	3E7[1]	Galα1-3[Fucα1-2]Galβ
Anti Lewis a	T174	Galβ1-3[Fucα1-4]GlcNAc
Anti sialyl Lewis a	KM231	NeuAcα2,3Galβ1-3[Fucα1-4]GlcNAc
Anti Lewis b	T218	Fucα1-2Galβ1-3[Fucα1-4]GlcNAc
Anti Lewis x	P12	Galβ1-4[Fucα1-3]GlcNAc
Anti sialyl Lewis x	KM93	NeuAcα2,3Galβ1-4[Fucα1-3]GlcNAc
Anti p^k	38-13	Galα1-4Galβ1-4Glc
Arachis hypogaea (Peanut)		Galβ1-3GalNAc; T-antigen
Erythrina cristagalli		Galβ1-4GlcNAc
Griffonia simplicifolia IB-4		αGal
Maackia amurensis		Neu5Acα2-3Galβ1-4GlcNAc/Glc
Sambucus nigra (Elder)		Neu5Acα2-6Gal/GalNAc
Ulex europaeus I (Furze)		Fucα1-2Gal
Vicia villosa IB_4 (Hairy vetch)		GalNAcα1-Ser/Thr; Tn-antigen

microtiter plate protocol of the BCA (bicinchoninic acid) Protein Assay Reagent (Pierce) according to the manufacturer's instructions. Samples were run in triplicate.

One-dimensional SDS-PAGE

SDS-PAGE was run by the method of Laemmli [24] with 5% stacking gels and 8% resolving gels using a vertical Mini-Protean II electrophoresis system (Bio-Rad, Hercules, CA, USA) and non-reducing conditions or with 4–12% Bis-Tris discontinuous polyacrylamide gels (NuPAGE; Invitrogen, California, USA) using a MES-buffer and non-reducing conditions. Before electrophoresis, individual milk samples were adjusted to a concentration of 3 mg/ml and, in order to avoid streaking, mixed with EDTA to a final concentration of 10 mM. A total protein amount of 11 μg was loaded per well. Separated proteins were visualized by a kit detecting glycosylated proteins by virtue of periodate oxidation of carbohydrate residues followed by conjugation to a fluorochrome emitting a bright green-fluorescent signal. Subsequently, the Ruby stain, which detects all proteins (Molecular Probes, Leiden, The Netherlands), was used.

Western blotting

Separated proteins were electrophoretically blotted onto nitrocellulose membranes (Invitrogen) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA). Membranes were blocked overnight in Phosphate-buffered saline with 0.2% Tween-20 (PBS-T) and 3% BSA, and were then incubated at room temperature for 1 h with antibodies diluted in the blocking buffer. Membranes were washed with PBS-T five times and incubated with HRP-conjugated secondary antibody. Bound secondary reagents were detected using the ECL Western blotting reagents (Amersham Biosciences) followed by exposure of the membrane on Hyperfilm ECL (Amersham Biosciences). The expression levels of various epitopes were assessed by densitometry of the films using the Quantity One software (Bio-Rad).

Results

As seen in Figure 1a, the size distribution and relative amounts of different proteins varied both within and between species. The main glycosylated proteins in human milk were tentatively characterized on the basis of molecular size as the mucins MUC-1 and MUC-X (>250 kDa), lactoferrin (79 kDa), butyrophilin (66–67 kDa), lactadherin (45–50 kDa), lactophorin (28 kDa) and lysozyme (14 kDa) (Figure 1b, lanes H). The main glycosylated proteins in bovine milk were, according to their apparent molecular masses, tentatively identified as MUC-1 (160–200 kDa), butyrophilin, lactadherin, lactophorin, κ-casein (20kDa) and lysozyme (Figure 1b, lanes C). In colostrum, a band that could correspond to orosomucoid (>400 kDa) was also seen (Figure 1b, lane CC). Goat (lanes G) and sheep (lanes S) showed a glycoprotein pattern similar to mature bovine milk, while the expression of pig milk glycoproteins (lanes P) were more similar to bovine colostrum. The glycoprotein expression pattern in milk from horse (lane Ho), dromedary (lane D) and rabbit (lane R) differed from the other species in terms of the number and amounts of glycoproteins expressed, with horse expressing the least of glycosylated proteins.

After separation by SDS-PAGE, the presence of carbohydrate epitopes on milk proteins were analysed by Western blotting using different lectins and carbohydrate-specific antibodies (Table 1).

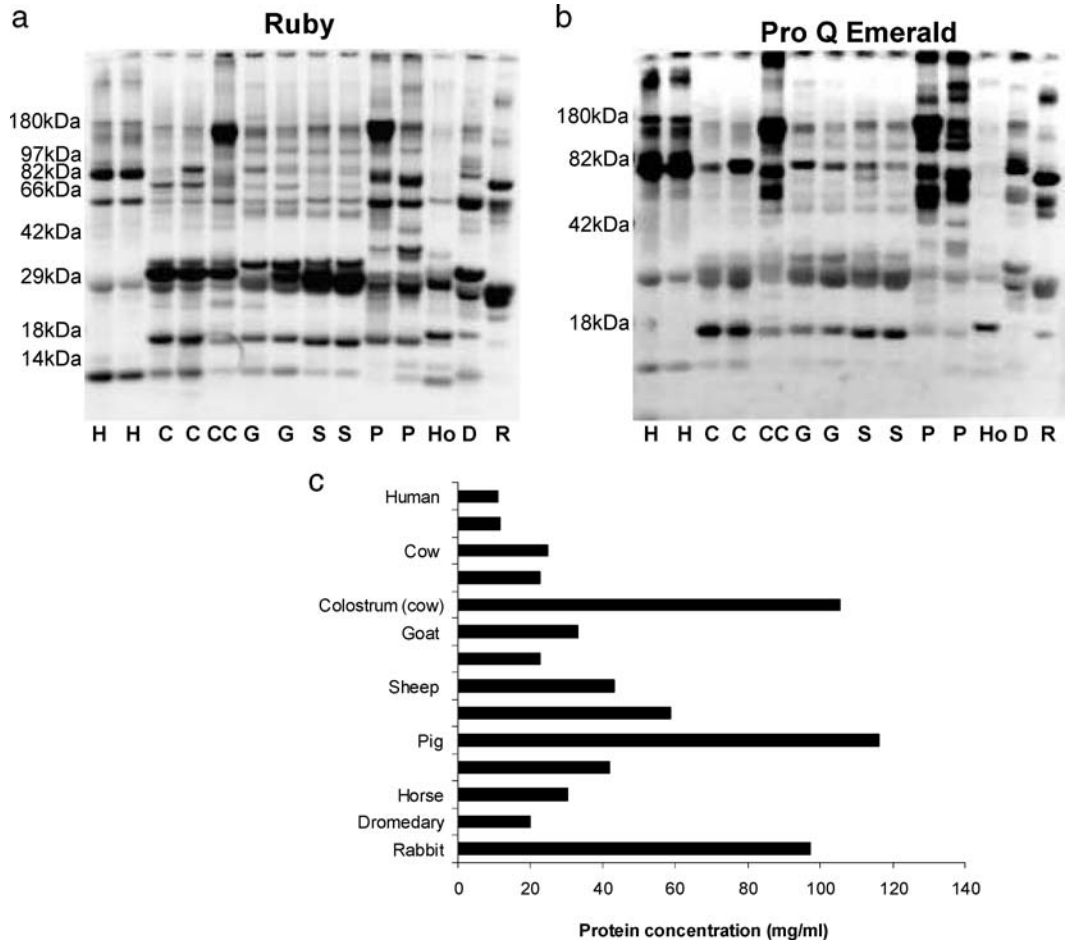

Figure 1. SDS-PAGE analysis of milk proteins showing all proteins as detected by staining with Ruby (a) and glycosylated proteins as detected by periodate oxidation and fluorochrome conjugation (b). Milk from man (lanes H), cow (lanes C; mature cow milk, and lane CC; cow colostrum), goat (lanes G), sheep (lanes S), pig (lanes P), horse (lane Ho), dromedary (lane D) and rabbit (lane R) were analyzed. 11 μg of total milk proteins were loaded per lane, and the total protein concentration in the milk of the various species is shown in c.

Lectin reactivities

Tn-, as defined by *V. villosa* reactivity, and T-antigens, as defined by *A. hypogaea* reactivity, were ubiquitously expressed and could be seen on a number of milk glycoproteins from all species investigated (Figure 2a and b). The Tn reactivity appeared stronger than the T-antigen reactivity. Type 2 structures, as defined by *E. cristagalli* reactivity, were expressed on milk glycoproteins from all species but rabbit (Figure 2c). However, goat and sheep in particular showed a considerably weaker staining. Expression was seen mainly on glycoproteins of molecular masses above 66 kDa, although pig and dromedary showed expression on a protein of around 45 kDa. *U. europaeus*-reactive epitopes could be detected on various milk glycoproteins in all species. Staining was especially strong for proteins from cow, goat, sheep and dromedary milk (Figure 2d). Sialylated epitopes were detected by the *M. amurensis* (Figure 3a) and the *S. nigra* (Figure 3b) lectins, with the former one being specific for α2-3-sialylated epitopes while the latter one is specific for α2-6-linked sialic acids. The expression of these epitopes seemed to be mutually exclusive to one another, although some overlapping reactivity was seen. For instance, milk proteins from cow, goat, sheep, dromedary and rabbit showed strong staining with *M. amurensis*, but weak staining with *S. nigra*. In contrast, milk proteins from man, cow colostrum and pig showed strong staining with *S. nigra*, but week staining with *M. amurensis*. Horse milk proteins were practically non-reactive with *M. amurensis*, while strong staining could be seen for one single protein with an apparent molecular weight of $>$220 kDa with the *S. nigra* lectin. The milk samples from the two pigs differed in their reactivity with the two sialic acid binding lectins. Milk proteins from both individuals showed strong staining with *S. nigra*, but only one of the individuals showed strong staining on a number of glycoproteins with *M. amurensis*. Terminal α-galactosylated epitopes could be detected on milk proteins of various sizes in all animal species using the

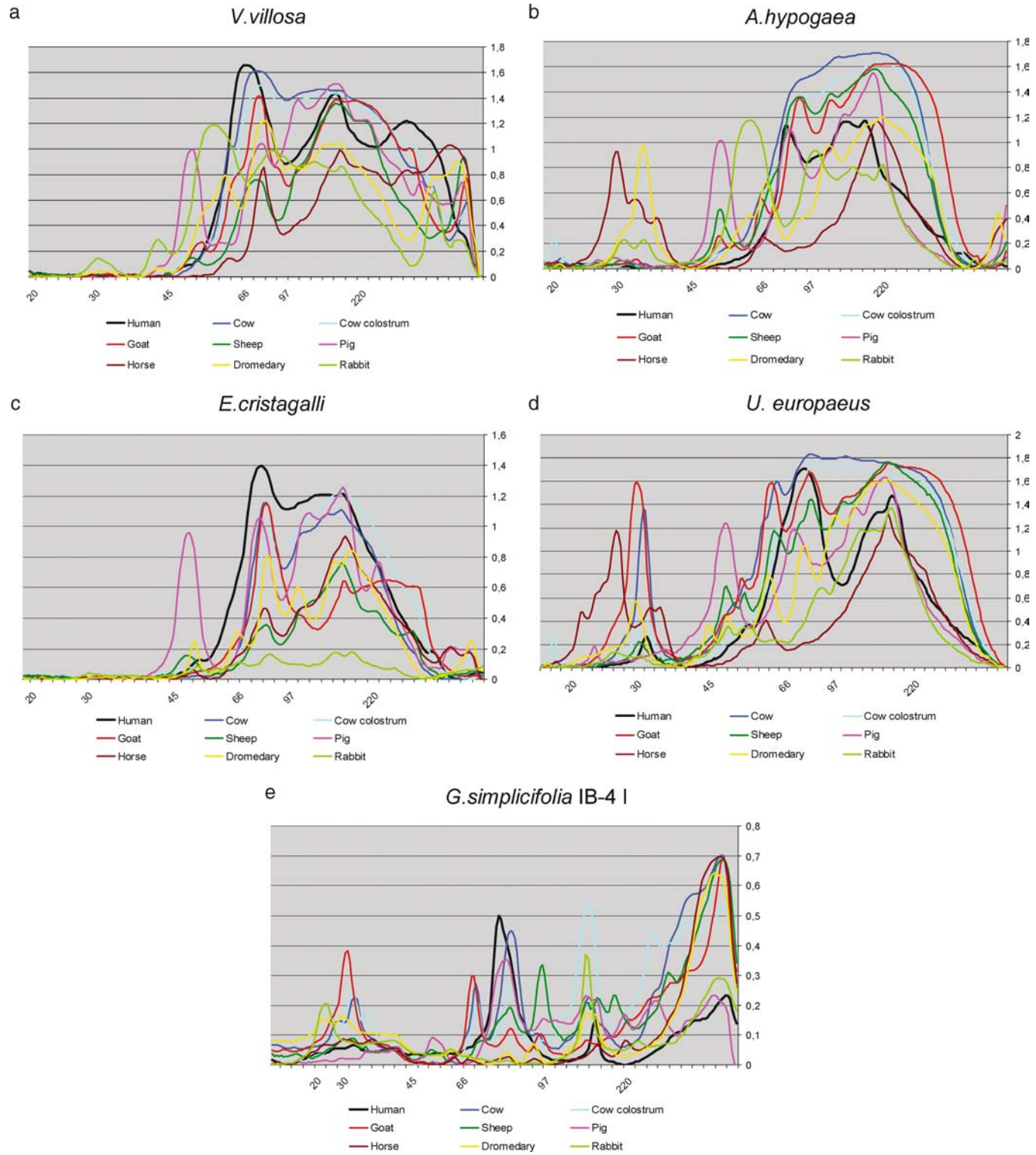

Figure 2. Graphs of the (a) *V. villosa*, (b) *A. hypogaea*, (c) *E. cristagalli*, (d) *U. europaeus* and (e) *G. simplicifolia* IB-4 I lectin reactivities. For simplicity, only one individual of each species were analyzed, using the same individual for each lectin. The expression levels of the carbohydrate epitopes for each species were defined by creating an intensity profile of the individual lanes for each Western blot film using the Quantity One software (Bio-Rad). For each lectin the optical density (Y-axis; pixels) of individual lanes was plotted against the Mr (X-axis, kDa) of detected milk proteins. Background noise in individual lanes on all films was subtracted using a rolling disk radius value of 50 pixels. Note that the optical density values (Y-axis) vary between the different lectins. 11 μg of total milk proteins were loaded per lane for the analyzed Western blots.

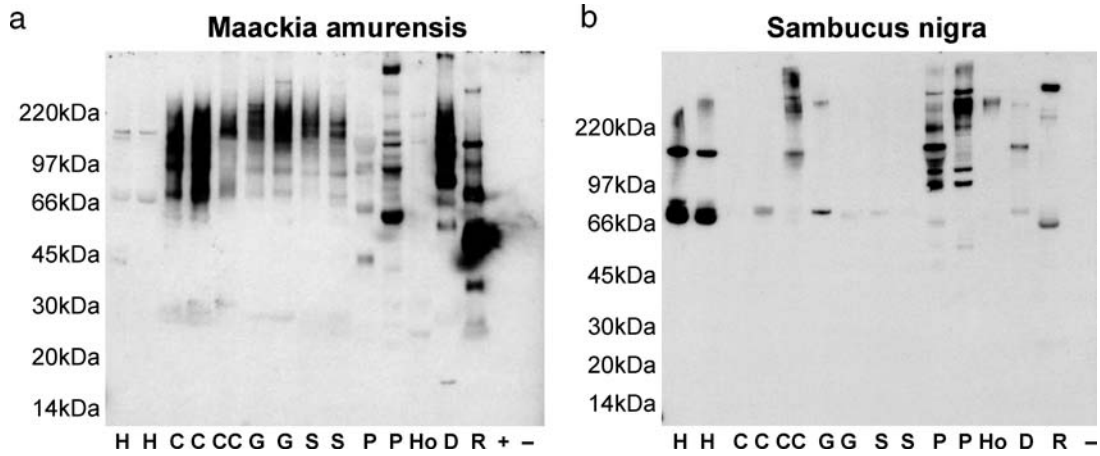

Figure 3. Western blotting of milk proteins probed with the lectins (a) *Maackia amurensis* (MAA) and (b) *Sambucus nigra*. For MAA, BSA conjugated with 3′Sialyl-LacNAc (100 ng) was used as a positive (+) control, while BSA (100 ng) was used as a negative (−) control for both lectins. Abbreviations; see Figure 1. 11 μg of total milk proteins were loaded per lane.

G. simplicifolia IB_4 lectin (Figure 2e). This was also the case for human milk proteins even though man lack α1,3-linked galactose except as part of the blood group B determinant. The *G. simplicifolia* reactivity in human milk may be explained by a terminal α-galactose linked to another carbon but carbon 3 in the penultimate sugar residue.

The ABH blood group antigens

Strong staining with the blood group H type 1 chain-specific antibody was seen on proteins of apparent molecular weights from 66 to 220 kDa in human milk. Also, strong bands of approximately 150, 70 and 30 kDa were seen for rabbit, pig and horse milk, respectively. The antibody also showed very weak reactivity with proteins of different sizes in milk from the other animals investigated (Figure 4a). Expression of the blood group H type 2 antigen, as detected by an anti-H type 2 chain-specific antibody, was seen in the milk of one of the pigs on proteins of apparent molecular weights of 150 and 50 kDa (Figure 4b). Staining was also seen for a protein of around 30 kDa in dromedary, rabbit, horse and cow, albeit very weakly for the latter two species. Man, one of the pigs and horse milk proteins showed reactivity with an anti-A antibody (Figure 4c). In human milk, staining was seen for proteins of apparent molecular weights of 150, 90 and 66 kDa. Reactivity with proteins of apparent molecular weights of 150 and 50 kDa were seen in the milk of one pig. In horse milk, binding was seen to proteins of apparent molecular weights of >220 and 66 kDa. Weak reactivity with the blood group B antigen, as detected by an anti-B antibody, was seen on two proteins of an apparent molecular weight of 220 and 150 kDa in sheep and pig, respectively (Figure 4d). As the pig is known to express the H and A blood group antigens only, this binding is most likely due to cross-reactivity with the Galili antigen (Galα1-3Galβ1-4GlcNAc), also known as the linear B saccharide.

The Lewis blood group antigens

Le^a reactivity was seen for human milk proteins of molecular masses between 50 and 200 kDa (Figure 5a). One of the individuals showed especially strong reactivity (H; second lane, Figure 5a). Weak staining was seen also for some animal milk proteins, especially pig, but this reactivity was consistent with non-specific secondary antibody binding (not shown). Sialyl-Le^a reactivity was also seen for human milk proteins of molecular masses between 50 and 200 kDa. However, this staining was, especially for one of the individuals, weaker than the Le^a reactivity (Figure 5b). Non-specific secondary antibody staining to some animal milk proteins could be seen here as well, but in contrast to the Le^a reactivity, the binding of the anti-sialyl-Le^a antibody to pig milk proteins of molecular masses between 100 and 200 kDa was specific. Human milk proteins of molecular masses of 45 kDa and larger showed a very strong staining with the anti-Le^b antibody. The same epitope, albeit with considerably weaker staining intensity, was detected on pig and horse milk proteins of apparent molecular masses of 150 and 50 kDa (pig) and >220 and 66 kDa (horse) (Figure 5c). Milk from the rest of the animals showed a weak staining on a few proteins of mainly low molecular weights (<50 kDa). Milk proteins from man and pig also showed a strong staining with an anti-Le^x antibody. This was seen on human proteins of apparent molecular masses of 45 kDa and larger, and on pig proteins of apparent molecular masses of 66–150 and 50 kDa. A significantly weaker staining was seen on several different proteins from cow, goat, sheep, horse, and rabbit (Figure 5d). In addition, milk proteins from man and pig showed staining with an anti-sialyl-Le^x antibody. These proteins have molecular weights of 90 kDa (human milk) as well as of 150, 66 and 50 kDa (pig milk). Horse milk expressed the sialyl-Le^x epitope on proteins of apparent molecular weights of >220 and 66 kDa. A very weak staining was also seen for a cow milk protein with an apparent molecular weight of 66 kDa (Figure 5e).

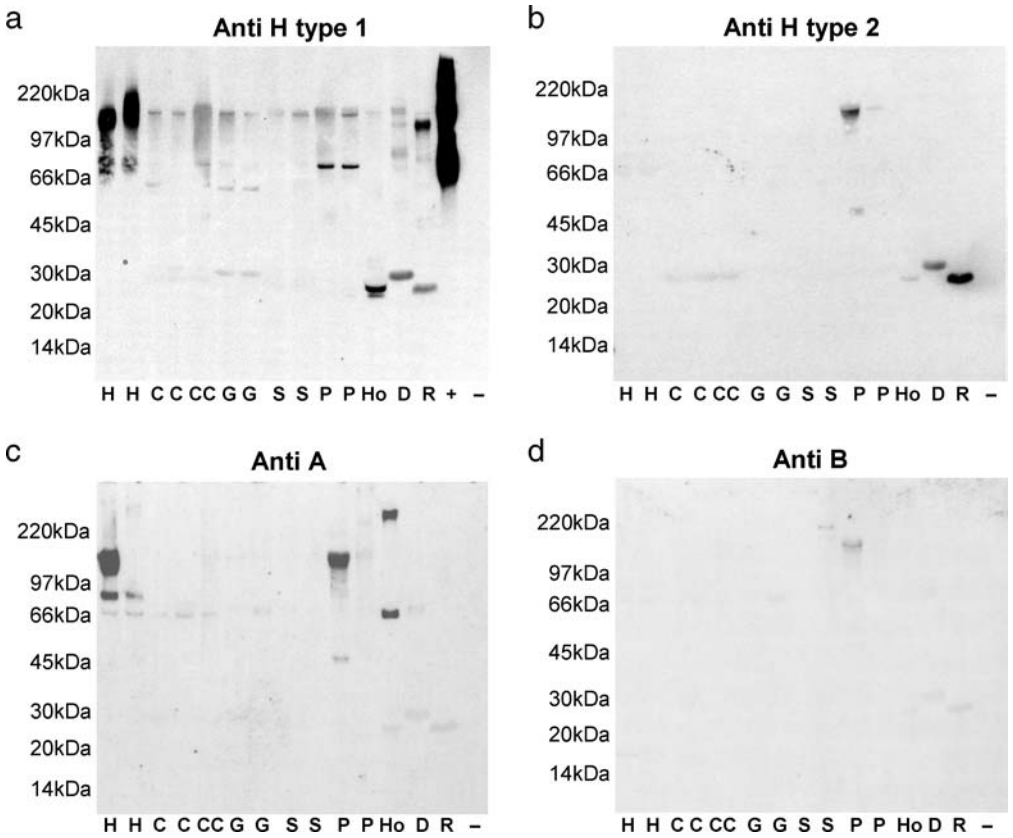

Figure 4. Western blotting of milk proteins probed with an anti-H type 1 antibody (a), H type 2 antibody (b), A antibody (c) and B antibody (d). BSA conjugated with H type 1 (100 ng) was used as a positive (+) control for the anti-H type 1 chain-specific antibody. BSA (100 ng) was used as a negative (−) control for all antibodies. For abbreviations see Figure 1. 11 μg of total milk proteins were loaded per lane.

The P blood group system

Anti-p^k, but not anti-P1, reactivity was seen on milk proteins from man and horse (not shown). These proteins and their reactivity with Shiga-like toxins and uropathogenic *E. coli* will be described elsewhere (A. Gustafsson *et al.*, manuscript in preparation).

Discussion

Our results showed that the determinants and the precursor chains of several carbohydrate epitopes known to mediate binding of pathogens were found in milk from all animals investigated. The blood group antigen H type 2 was expressed on milk proteins from cow, pig, horse, dromedary and rabbit. Furthermore, Neu5Acα2-3LacNAc, the epitope recognized by the lectin *M. amurensis*, and also the precursor for sialyl-Le^x, was expressed on milk proteins from all species investigated, with especially strong staining on cow, dromedary and rabbit milk proteins. Several different fucosyltransferases (FUT III-VII) can biosynthesize sialyl-Le^x from Neu5Acα2-3LacNAc [25–27]. The Le^b epitope is produced by the action of FUT III, an enzyme adding fucose in an α1-4 linkage to the GlcNAc residue in H type 1 [28]. The blood group antigen H type 1, as well as its precursor, the type 1 chain, was expressed on milk proteins from all animals investigated. Transgenic expression of the above mentioned fucosyltransferase genes in the mammary gland of these animals could therefore result in the production of the Lewis blood group antigens Le^b and sialyl-Le^x, which are known binding epitopes for *Helicobacter pylori*. This pathogen infects some 50% of the world population and in 10% of these cases, infection results in the development of peptic ulcer disease, mucosa-associated lymphoid-tissue (MALT) lymphoma or gastric adenocarcinoma [29,30]. Both human and bovine milk has been shown to have protective properties against *H. pylori* [6,31–33], although this protection was not linked to expression of Le^b and sialyl-Le^x carbohydrate epitopes. Human, pig and horse milk proteins were found to also express the Le^b and sialyl-Le^x carbohydrate epitopes. The binding properties of *H. pylori* to different glycoconjugates have been extensively studied, and a number of binding specificities identified [34–39]. *H. pylori* binding to different carbohydrate structures are mediated by adhesins expressed on its surface. Two of these, the BabA and the SabA adhesins have been purified and characterized. The BabA adhesin mediates binding to Le^b [34,40] while the SabA adhesin preferentially binds sialyl-Le^x [41,42]. The

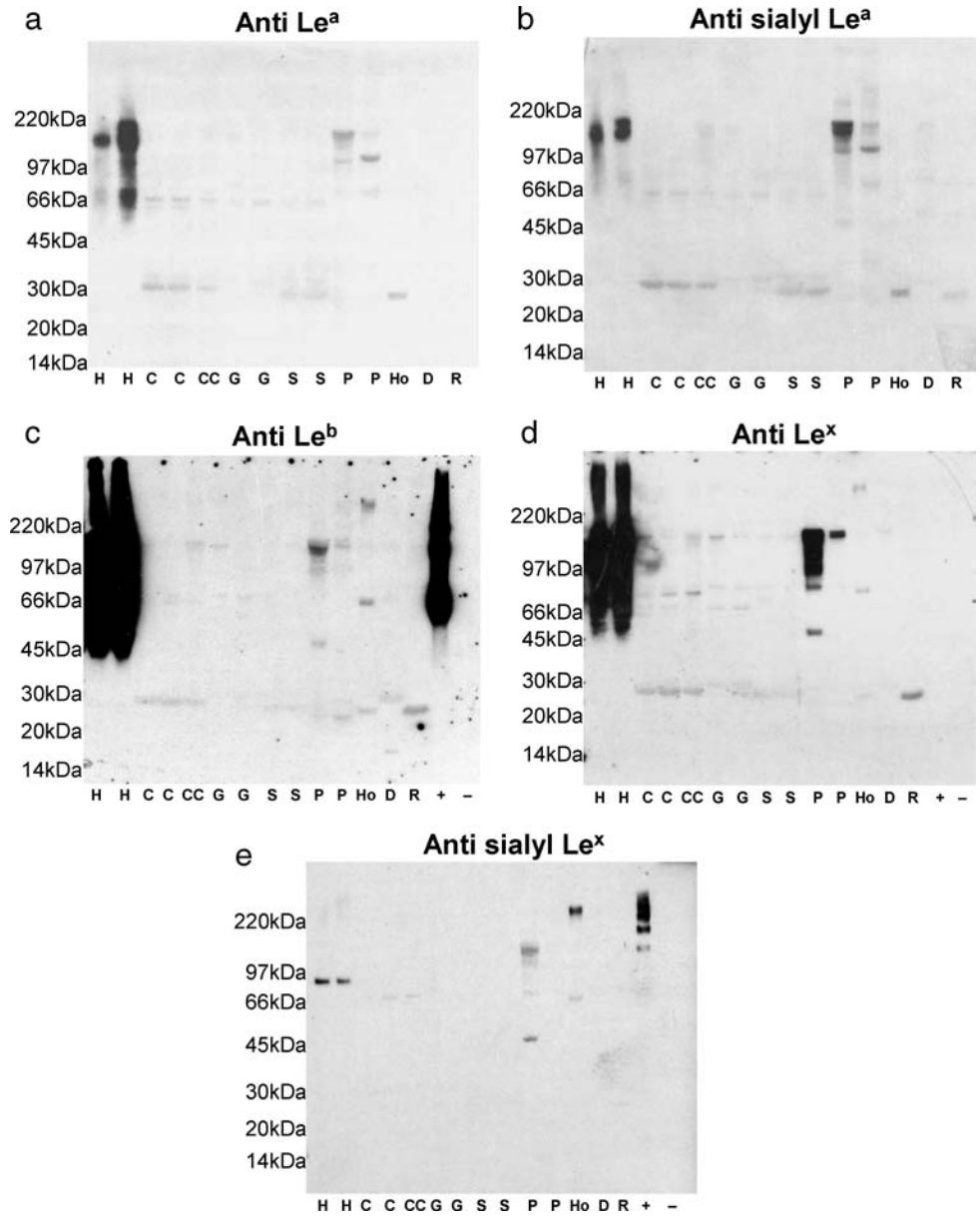

Figure 5. Western blotting of milk proteins probed with an anti-Le^a antibody (a), sialyl-Le^a antibody (b), Le^b antibody (c), Le^x antibody (d), and sialyl-Le^x antibody (e). For the anti-Le^b, anti-Le^x, and the anti-sialyl Le^x antibodies, Le^b-BSA (100 ng), Le^x-BSA (100 ng) and sialyl Le^x-BSA (100 ng), respectively, were used as positive (+) controls and BSA (100 ng) as a negative (−) control. For abbreviations, see Figure 1. 11 μg of total milk proteins were loaded per lane.

expression of Le^b and sialyl-Le^x on milk proteins from different pig breeds and its potentially inhibitory effect on *H. pylori* colonization is currently being investigated (A. Gustafsson *et al.*, manuscript in preparation).

Enteropathogenic *Escherichia coli* (EPEC) is in developing countries one of the main causes of infantile diarrhea and death in children less than five years of age [43]. Localized adherence of this bacterium to HEp-2 cells was inhibited by fucosylated human milk oligosaccharides *e.g.* H type 1, Le^a and Le^x [44]. The heat-stable toxin (ST) of enterotoxic *E. coli* (ETEC) is also known to cause infantile diarrhea, although its pathogenic effect is less aggressive compared to EPEC [45]. Suckling mice were protected from ST by a minor, non-identified fucosylated oligosaccharide in human milk [46]. Also, infants whose mothers expressed more 2-linked (H type 1 and 2) than non 2-linked (Le^a and Le^x) fucosyloligosaccharides in their milk exhibited higher protection against ST [47]. Finally, the expression of the H type 2 blood group antigen in human milk was shown to inhibit binding and infection by *Campylobacter jejuni*, another common cause of infantile diarrhea [18]. All of these epitopes were identified on human milk proteins, but some of the epitopes were also clearly expressed in animal milk, especially in

pig, but also in horse, dromedary and rabbit milk. S-fimbriated *E. coli*, a pathogen causing sepsis and meningitis in neonates, have been shown to recognize the Neu5Acα2-3Galβ1-4 sequence in oligosaccharides [48]. The same structure could be recognized on human milk glycoproteins and brain microvascular endothelial cells [9,49]. As stated above, this epitope was present in milk from all species (as part of the structure recognized by the lectin *Maackia amurensis*), although staining of man and horse milk proteins was very weak.

For the production of large volumes of milk from genetically modified animals, cow would be the immediate choice considering its already established status as a milk producer in the industrialized world. However, for production of certain carbohydrate epitopes, transgenic expression of several different glycosyltransferases might be necessary. It remains to be shown whether this would actually result in production of the desired carbohydrate epitopes, if pathogen binding can be achieved, and if the amounts produced of specific carbohydrate epitopes are enough for pathogen inhibition. These factors will depend on the capability of the expressed glycosyltransferases to utilize preexisting milk glycoconjugates, but also on the binding preferences of the pathogen considering the fact that inner saccharide core structures have been shown to affect the conformation of terminal carbohydrate epitopes.

In conclusion, we have shown that the carbohydrate epitopes, and/or their precursor structures, recognized by different pathogens were expressed on milk proteins from several of the animals investigated. The study is of importance as it gives some general information regarding the carbohydrate structures expressed on milk proteins from different animals. However, the expression of carbohydrate epitopes can be both individual- and breed-specific, therefore more specific information regarding the carbohydrate epitopes expressed in a given breed of a particular species will necessarily have to include a larger number of animals. The results presented here may provide some initial guidance as to the animal of choice for a potential establishment of milk as a therapeutic agent for pathogen inhibition.

Acknowledgments

This work was supported by the Swedish Research Council and the National Research Fund of Hungary (OTKA T035209). J.H. holds a position within the program "Glycoconjugates in Biological Systems" financed by the Swedish Foundation for Strategic Research.

References

1 Ofek I, Sharon N, Adhesins as lectins: Specificity and role in infection, *Curr Top Microbiol Immunol* **151**, 91–113 (1990).
2 Hamosh M, Protective function of proteins and lipids in human milk, *Biol Neonate* **74**(2), 163–76 (1998).
3 Newburg DS, Neubauer SH, *Carbohydrates in Milks: Analysis, Quantities, and Significance* (Academic Press, Inc, 1995), pp. 273–349.
4 Burgoyne RD, Duncan JS, Secretion of milk proteins, *J Mammary Gland Biol Neoplasia* **3**(3), 275–86 (1998).
5 Mather IH, Keenan TW, Origin and secretion of milk lipids, *J Mammary Gland Biol Neoplasia* **3**(3), 259–73 (1998).
6 Stromqvist M, Falk P, Bergstrom S, Hansson L, Lonnerdal B, Normark S, Hernell O, Human milk kappa-casein and inhibition of Helicobacter pylori adhesion to human gastric mucosa, *J Pediatr Gastroenterol Nutr* **21**(3), 288–96 (1995).
7 Andersson B, Porras O, Hanson LA, Lagergard T, Svanborg-Eden C, Inhibition of attachment of Streptococcus pneumoniae and Haemophilus influenzae by human milk and receptor oligosaccharides, *J Infect Dis* **153**(2), 232–7 (1986).
8 Peterson JA, Patton S, Hamosh M, Glycoproteins of the human milk fat globule in the protection of the breast-fed infant against infections, *Biol Neonate* **74**(2), 143–62 (1998).
9 Schwertmann A, Schroten H, Hacker J, Kunz C, S-fimbriae from Escherichia coli bind to soluble glycoproteins from human milk, *J Pediatr Gastroenterol Nutr* **28**(3), 257–63 (1999).
10 Holmgren J, Svennerholm AM, Lindblad M, Receptor-like glycocompounds in human milk that inhibit classical, El Tor Vibrio cholerae cell adherence (hemagglutination), *Infect Immun* **39**(1), 147–54 (1983).
11 Newburg DS, Ashkenazi S, Cleary TG, Human milk contains the Shiga toxin and Shiga-like toxin receptor glycolipid Gb3, *J Infect Dis* **166**(4), 832–6 (1992).
12 Herrera-Insua I, Gomez HF, Diaz-Gonzalez VA, Chaturvedi P, Newburg DS, Cleary TG, Human milk lipids bind Shiga toxin, *Adv Exp Med Biol* **501**, 333–9 (2001).
13 Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KH, Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts, *Science* **278**(5346), 2130–3 (1997).
14 Brink MF, Bishop MD, Pieper FR, Developing efficient strategies for the generation of transgenic cattle which produce biopharmaceuticals in milk, *Theriogenology* **53**(1), 139–48 (2000).
15 Clark AJ, The mammary gland as a bioreactor: Expression, processing, and production of recombinant proteins, *J Mammary Gland Biol Neoplasia* **3**(3), 337–50 (1998).
16 Karatzas CN, Designer milk from transgenic clones, *Nat Biotechnol* **21**(2), 138–9 (2003).
17 Prieto PA, Mukerji P, Kelder B, Erney R, Gonzalez D, Yun JS, Smith DF, Moremen KW, Nardelli C, Pierce M, *et al.* Remodeling of mouse milk glycoconjugates by transgenic expression of a human glycosyltransferase, *J Biol Chem* **270**(49), 29515–9 (1995).
18 Ruiz-Palacios GM, Cervantes LE, Ramos P, Chavez-Munguia B, Newburg DS, Campylobacter jejuni binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection, *J Biol Chem* **278**(16), 14112–20 (2003).
19 Kunz C, Rudloff S, Schad W, Braun D, Lactose-derived oligosaccharides in the milk of elephants: Comparison with human milk, *Br J Nutr* **82**(5), 391–9 (1999).
20 Mather IH, A review and proposed nomenclature for major proteins of the milk-fat globule membrane, *J Dairy Sci* **83**(2), 203–47 (2000).
21 Mammen M, Choi S, Whitesides G, Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors, *Angew Chem Int Ed* **37**, 2754–2794 (1998).

22 Lindhorst TK, Kieburg C, Krallmann-Wenzel U. Inhibition of the type 1 fimbriae-mediated adhesion of Escherichia coli to erythrocytes by multiantennary alpha-mannosyl clusters: The effect of multivalency, *Glycoconj J* **15**(6), 605–13 (1998).
23 Totani K, Kubota T, Kuroda T, Murata T, Hidari KI, Suzuki T, Suzuki Y, Kobayashi K, Ashida H, Yamamoto K, Usui T, Chemoenzymatic synthesis and application of glycopolymers containing multivalent sialyloligosaccharides with a poly(L-glutamic acid) backbone for inhibition of infection by influenza viruses, *Glycobiology* **13**(5)315–26 (2003).
24 Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **227**(259), 680–5 (1970).
25 Kimura H, Shinya N, Nishihara S, Kaneko M, Irimura T, Narimatsu H, Distinct substrate specificities of five human alpha-1,3-fucosyltransferases for in vivo synthesis of the sialyl Lewis x and Lewis x epitopes, *Biochem Biophys Res Commun* **237**(1), 131–7 (1997).
26 Grabenhorst E, Nimtz M, Costa J, Conradt HS, In vivo specificity of human alpha1,3/4-fucosyltransferases III-VII in the biosynthesis of LewisX and Sialyl LewisX motifs on complex-type *N*-glycans. Coexpression studies from bhk-21 cells together with human beta-trace protein, *J Biol Chem* **273**(47), 30985–94 (1998).
27 Huang MC, Laskowska A, Vestweber D, Wild MK, The alpha (1,3)-fucosyltransferase Fuc-TIV, but not Fuc-TVII, generates sialyl Lewis X-like epitopes preferentially on glycolipids, *J Biol Chem* **277**(49), 47786–95 (2002).
28 Prieels JP, Monnom D, Dolmans M, Beyer TA, Hill RL, Copurification of the Lewis blood group *N*-acetylglucosaminide alpha 1 goes to 4 fucosyltransferase, an *N*-acetylglucosaminide alpha 1 goes to 3 fucosyltransferase from human milk, *J Biol Chem* **256**(20), 10456–63 (1981).
29 Blaser MJ, The bacteria behind ulcers, *Sci Am* **274**(2), 104–7 (1996).
30 Crespo A, Suh B, Helicobacter pylori infection: Epidemiology, pathophysiology, and therapy, *Arch Pharm Res* **24**(6), 485–98 (2001).
31 Clyne M, Thomas J, Weaver L, Drumm B, In vitro evaluation of the role of antibodies against Helicobacter pylori in inhibiting adherence of the organism to gastric cells, *Gut* **40**(6), 731–8 (1997).
32 Hata Y, Kita T, Murakami M, Bovine milk inhibits both adhesion of Helicobacter pylori to sulfatide and Helicobacter pylori-induced vacuolation of vero cells, *Dig Dis Sci* **44**(8), 1696–702 (1999).
33 Wang X, Hirmo S, Willen R, Wadstrom T, Inhibition of Helicobacter pylori infection by bovine milk glycoconjugates in a BAlb/cA mouse model, *J Med Microbiol* **50**(5), 430–5 (2001).
34 Borén T, Falk P, Roth KA, Larson G, Normark S, Attachment of Helicobacter pylori to human gastric epithelium mediated by blood group antigens, *Science* **262**(5141), 1892–5 (1993).
35 Falk P, Roth KA, Boren T, Westblom TU, Gordon JI, Normark S, An in vitro adherence assay reveals that Helicobacter pylori exhibits cell lineage-specific tropism in the human gastric epithelium, *Proc Natl Acad Sci USA* **90**(5), 2035–9 (1993).
36 Miller-Podraza H, Milh MA, Teneberg S, Karlsson KA, Binding of Helicobacter pylori to sialic acid-containing glycolipids of various origins separated on thin-layer chromatograms, *Infect Immun* **65**(6), 2480–2 (1997).
37 Teneberg S, Miller-Podraza H, Lampert HC, Evans DJ, Jr., Evans DG, Danielsson D, Karlsson KA, Carbohydrate binding specificity of the neutrophil-activating protein of Helicobacter pylori, *J Biol Chem* **272**(30), 19067–71 (1997).
38 Angstrom J, Teneberg S, Milh MA, Larsson T, Leonardsson I, Olsson BM, Halvarsson MO, Danielsson D, Naslund I, Ljungh A, Wadstrom T, Karlsson KA, The lactosylceramide binding specificity of Helicobacter pylori, *Glycobiology* **8**(4), 297–309 (1998).
39 Roche N, Larsson T, Angstrom J, Teneberg S, Helicobacter pylori-binding gangliosides of human gastric adenocarcinoma, *Glycobiology* **11**(11), 935–44 (2001).
40 Ilver D, Arnqvist A, Ogren J, Frick IM, Kersulyte D, Incecik ET, Berg DE, Covacci A, Engstrand L, Boren T, Helicobacter pylori adhesin binding fucosylated histo-blood group antigens revealed by retagging, *Science* **279**(5349), 373–7 (1998).
41 Syder AJ, Guruge JL, Li Q, Hu Y, Oleksiewicz CM, Lorenz RG, Karam SM, Falk PG, Gordon JI, Helicobacter pylori attaches to NeuAc alpha 2,3Gal beta 1,4 glycoconjugates produced in the stomach of transgenic mice lacking parietal cells, *Mol Cell* **3**(3), 263–74 (1999).
42 Mahdavi J, Sonden B, Hurtig M, Olfat FO, Forsberg L, Roche N, Angstrom J, Larsson T, Teneberg S, Karlsson KA, Altraja S, Wadstrom T, Kersulyte D, Berg DE, Dubois A, Petersson C, Magnusson KE, Norberg T, Lindh F, Lundskog BB, Arnqvist A, Hammarstrom L, Boren T, Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation, *Science* **297**(5581), 573–8 (2002).
43 Clarke SC, Haigh RD, Freestone PP, Williams PH, Virulence of enteropathogenic Escherichia coli, a global pathogen, *Clin Microbiol Rev* **16**(3), 365–78 (2003).
44 Cravioto A, Tello A, Villafan H, Ruiz J, del Vedovo S, Neeser JR, Inhibition of localized adhesion of enteropathogenic Escherichia coli to HEp-2 cells by immunoglobulin and oligosaccharide fractions of human colostrum and breast milk, *J Infect Dis* **163**(6), 1247–55 (1991).
45 Gaastra W, Svennerholm AM, Colonization factors of human enterotoxigenic Escherichia coli (ETEC), *Trends Microbiol* **4**(11), 444–52 (1996).
46 Newburg DS, Pickering LK, McCluer RH, Cleary TG, Fucosylated oligosaccharides of human milk protect suckling mice from heat-stabile enterotoxin of Escherichia coli, *J Infect Dis* **162**(5), 1075–80 (1990).
47 Newburg DS, Ruiz-Palacios GM, Altaye M, Chaturvedi P, Meinzen-Derr J, Guerrero Mde L, Morrow AL, Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants, *Glycobiology* **14**(3), 253–63 (2004).
48 Korhonen TK, Vaisanen-Rhen V, Rhen M, Pere A, Parkkinen J, Finne J, Escherichia coli fimbriae recognizing sialyl galactosides, *J Bacteriol* **159**(2), 762–6 (1984).
49 Prasadarao NV, Wass CA, Kim KS, Identification and characterization of S fimbria-binding sialoglycoproteins on brain microvascular endothelial cells, *Infect Immun* **65**(7), 2852–60 (1997).

Received 15 October 2004; revised 14 January 2005; accepted 20 January 2005