Conglutinin is not specific to cattle

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ABSTRACT: Conglutinin is a high-molecular-weight mammalian lectin which binds in a calcium-dependent manner to cell-surface-bound complement fragment iC3b, yeast cell-wall extract and terminal non-reducing N-acetyl-D-glucosamine, mannose and fucose residues. This protein, originally detected in bovine serum, belongs to the family of collectins, which are effector molecules in innate immunity. Conglutinin appears to play an important role in defence mechanisms, showing antiviral and antibacterial activity. We have characterized the electrophoresis profile of bovine serum conglutinin and used Western blotting to compare profiles of this lectin derived from the sera of different breeds of cattle. The profile of non-reduced conglutinin is characterised by many bands with molecular masses ranging from 34 to 630 kDa. Reduced lectin takes the form of three main bands with molecular masses of 41, 47 and 96 kDa. We show that conglutinin is present not only in adult bovine serum, but also in foetal bovine serum, colostrum and milk. The sera of sheep, goats, gnu antelopes and deer, as well as some non-ruminant species such as llamas, horses, boars, pigs and humans, contain proteins which have similar antigenicity to that of bovine conglutinin. These reacted with monoclonal and polyclonal antibodies specific for bovine conglutinin under reducing and non-reducing conditions in Western blotting. The protein profiles of bison and swine lectin were observed to be particularly similar to bovine conglutinin.

Keywords: animal collectins; lectins; conglutinin-like proteins; innate immunity

Conglutinin was originally described as a bovine serum component capable of agglutinating erythrocytes opsonized with antibodies and complement (Bordet and Gay, 1906). This unique process, called conglutination, is based on the binding of conglutinin to the carbohydrate moiety on the alpha chain of the complement degradation product iC3b (Laursen et al., 1994), and has been used to estimate the conglutinin titre in serum (Ingram and Mitchell, 1971a). It has been suggested that conglutinin does not occur only in bovine serum. Conglutinating activity is also displayed by the sera of many African ruminants (Lachmann, 1967), swine (Tlaskalova and Barta, 1968) and sheep (Jonas and Stankiewicz, 1984).

Conglutinin has been classified as a member of the subfamily of C-type (Ca^{2+}-dependent) lectins termed collectins. These generally oligomeric proteins occur commonly in mammals and chickens (Hogenkamp et al., 2006), but they have been found also in fish (Kania et al., 2010) and in invertebrates (tunicates) (Green et al., 2006). All collectins contain in their structure collagen-like domains and carbohydrate recognition domains (CRDs), and are able to bind sugar residues in a calcium-dependent manner. Besides conglutinin, the mammalian collectins include mannan binding protein (MBP), surfactant proteins A (SP-A) and D (SP-D) and bovine collectin 43 (CL-43). In recent years much attention has been devoted to collectins, focusing on their role in innate immunity. It is believed that by binding to microbial surface carbohydrates these lectins can induce aggregation and prevent the spread of pathogens. Moreover, they are able to mediate microbial destruction by stimulating phagocytosis or activating the complement system (van de Wetering et al., 2004).

There is little data concerning the biological function of conglutinin, but it must play an important role in organisms because the serum concentration of this lectin varies with the season of the year, diet, stage of the reproductive cycle, and infections (Dec and Wernicki, 2006). It has been suggested that the low concentration of serum conglutinin during

acute infections such as pneumonia or metritis is caused by its absorption by activated complement component iC3b or directly by microorganisms (Ingram and Mitchell, 1971b). Conglutinin has also been shown to exhibit some antibacterial (Friis-Christiansen et al., 1990) and antiviral properties (Hartshorn et al., 1993; Reading et al., 1998).

The purpose of this study was to determine the electrophoresis profiles of conglutinin obtained from different breeds of cattle and to ascertain whether conglutinin is indeed present in bovine colostrum, milk and precolostral calf serum as well as in the sera of some other ruminant and non-ruminant species.

MATERIAL AND METHODS

Isolation of lectin fractions from sera and milk

Serum samples. Blood samples were collected by jugular venipuncture from adult cattle (two to three years old), goats, sheep, other livestock (horses, swine), wild animals (bison), and zoo animals (deer, llamas, gnu antelopes, boars). Human peripheral blood was obtained from the Regional Centre for Blood Donation and Haemotherapy in Lublin. In the case of the cattle we chose a few dairy breeds – Polish Lowland, black and white and red and white Holstein-Friesian – and a few meat breeds – Piemontese, Limousine, Aberden Angus and Charolaise. The goats used were Boer goat, Saanen goat, White Improved goat and Syrian goat, and the sheep breeds were Polish Lowland sheep of the Uhruska variety, Suffolk, Merino and synthetic prolific meat line sheep (BCP). The BCP line was created in Bezek using Polish Lowland sheep of the Uhruska variety, prolific breeds (Romanowska, Finnish, Okulska, Booroola), Berrichone du Cher and Charolaise (Pieta and Patkowski, 2009). All animals were clinically healthy.

We also used foetal bovine sera (FBS) taken from two newborn precolostral calves and commercial FBS collected in the USA (Sigma-Aldrich, Poland, F6178), Australia (Invitrogen, USA, 10099-133), South America (Thermo Fisher Scientific, USA, SV30160.02) and EU-approved countries (Biochrom, UK, S0113).

Colostrum and milk samples. Bovine colostrum from first milking and milk was collected from Polish Black-and-White Lowland cows and stored frozen at –20 °C until analysis. Fat from the samples was removed before use by centrifugation at 4000 × g, 30 min.

Buffers and solutions

Tris buffered saline (TBS): 10mM Tris, 140mM NaCl, 0.05% emulfogen (polyoxyethylene 10 tridecyl ether, Sigma-Aldrich, Poland), pH 7.4; TBS-Ca: TBS with 5mM $CaCl_2$; TBS-Ca-1M NaCl: TBS-Ca containing a total of 1M NaCl; TBS-EDTA: TBS with 5mM EDTA.

About 25 ml of serum, colostrum or milk was incubated with 5 ml of chromatographic medium mannan-agarose for 3 h at 4 °C. The medium was washed several times, first with TBS-Ca-1MNaCl buffer and then with TBS-Ca. The lectins were eluted with TBS-EDTA. The protein concentration in the fractions obtained was estimated according to the method described by Bradford (1976) using bovine serum albumin (BSA) as the standard. Lectin solutions with the addition of protease inhibitor cocktail (Sigma-Aldrich, Poland) were stored at –20 °C. The presence of conglutinin in the obtained fractions was detected in Western blotting using monoclonal and polyclonal antibodies specific for bovine conglutinin.

Purification of bovine conglutinin

Conglutinin was purified according to the method developed by Krogh-Meibom et al. (2010), with some modifications. Affinity chromatography was used in the first stage and ion-exchange chromatography in the second. Bovine serum was batch-incubated with TSK beads (Toyopearl HW-75F, Tosh Bioscience, Japan) for 2.5 h at 37 °C in a glass flask. The serum-treated TSK beads were packed under flow on a FPLC system (Bio-Rad, Poland). The protein fraction eluted with TBS-EDTA was applied to an ion-exchange column (Macro-Prep High-Q Support, Bio-Rad, Poland) and the proteins retained were separated by running a linear gradient of NaCl.

Electrophoresis

Electrophoresis was performed on a 3 to 20% polyacrylamide gradient gel as described by Laemmli (1970) and was carried out at a constant voltage of 100 V. Protein samples were heated at 99 °C for 5 min in SDS-PAGE sample buffer for non-reduced

samples and in SDS-PAGE sample buffer containing 50mM 2-mercaptoethanol for reduced samples. Approximately 4 µg of total protein was applied to each lane. PageRuler^{TM} Prestained Protein Ladder (Thermo Fisher Scientific, Fermentas, USA) with a mass range of 11 to 170 kDa or 11 to 250 kDa was used as a molecular weight standard.

Immunoblotting

Following SDS-PAGE, gels were equilibrated in blotting buffer (12mM Tris, 96mM glycine, 20% methanol, pH 8.3) and then the proteins were electroblotted (Mini Trans-Blot cell, Bio-Rad, Poland) onto polyvinylidene difluoride (PVDF) membranes as described by Towbin et al. (1979). Transfer was conducted at 100 V for 2 h. After blocking with low-fat milk protein, the membranes were incubated overnight with monoclonal anti-conglutinin antibodies (mab 263-01, Abcam) diluted 1 : 1000 or with rabbit antiserum specific for conglutinin diluted 1 : 200 in TBST. Polyclonal peroxidase labeled rabbit anti-mouse IgG (Abcam, UK) or goat anti-rabbit IgG (Jackson ImmunoResearch, USA) diluted 1 : 5000 in TBST (time of incubation 2 h) were used as secondary antibodies. To induce a colorimetric reaction, 1.4 chloronaphthol (Bio-Rad, Poland) was used as a substrate.

Densitometric analysis of the protein bands obtained was carried out using the Gel Doc 2000 gel documentation system (Bio-Rad, Poland) and the computer program Quantity One (Bio-Rad, Poland).

Preparation of anti-conglutinin serum

For rabbit immunization bovine conglutinin purified in a two-step chromatographic procedure was mixed with incomplete Freunds adjuvant (Sigma-Aldrich, Poland) at 1 : 1 vol/vol. The rabbit was immunized subcutaneously four times every other week with 50 µg of conglutinin.

RESULTS

Conglutinin purification and preparation of anti-conglutinin rabbit serum

As monoclonal antibodies specific for bovine conglutinin react only with native and non-reduced protein, rabbit antiserum was used to detect reduced conglutinin. A comparative characterization of the conglutinin protein profiles in gel and on PVDV membranes indicated a high level of purity for the protein obtained, which in SDS-PAGE under non-reduced conditions takes the form of a ladder composed of many bands with molecular masses ranging from 34 to 630 kDa. The rabbit polyclonal antibodies reacted with both non-reduced and reduced conglutinin, while the rabbit serum collected before immunization did not exhibit such reactivity. No differences were observed between the protein profiles for non-reduced conglutinin obtained in Western blotting using monoclonal and polyclonal antibodyies (Figure 1).

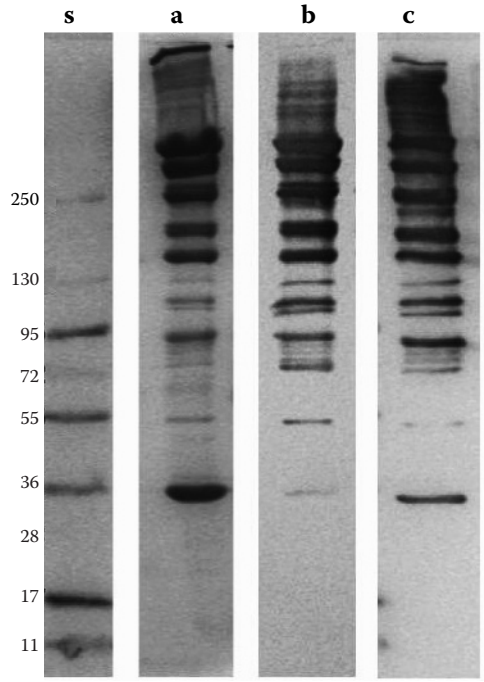

Figure 1. Conglutinin purified from bovine serum using a two-step chromatographic method. **a** = SDS-PAGE analysis of non-reduced conglutinin in 3 to 20% polyacrylamide gel, Coomassie brilliant blue R-250; **b** = Western blotting – reaction of purified non-reduced conglutinin with monoclonal antibodies (mab 236-01, Abcam); **c** = Western blotting – reaction of purified non-reduced conglutinin with anti-conglutinin rabbit serum; **s** = molecular weight standard (Fermentas)

Detection of conglutinin and lectins of similar antigenicity in immunoblotting

To determine the range of occurrence of conglutinin in animals, experiments were conducted on

sera from different breeds of dairy and meat cattle, colostrum and milk from cows, foetal bovine serum, sera from goats, sheep, and selected ruminant and non-ruminant species living in zoos or in the wild, and also from humans. Lectins were isolated from the material collected using the chromatographic medium mannan-agarose. Conglutinin and serum lectins of similar antigenicity were identified in Western blotting under non-reduced and reduced conditions, using monoclonal and polyclonal antibodies specific for bovine conglutinin.

Bovine conglutinin

Densitometric analysis of the images obtained in immunoblotting showed no significant differences in the protein profiles of non-reduced conglutinin derived from different cattle breeds. Reactions were observed in all the breeds studied between monoclonal antibodies and fractions with molecular masses of 34, 79, 89, 113, 127, 153, 175, 250, 280, 338, 387, 487, 570 and 630 kDa. The strongest reactions were with bands of 153, 175, 250, 280, 338 and 387 kDa. In some breeds weak reactions were additionally observed at 39, 42, 51, 64 and 107 kDa (Figure 2A).

Reduced conglutinin derived from the cattle breeds studied, identified using rabbit antiserum, took the form of three bands with molecular masses of 41, 47 and 96 kDa. In most cases weak reactions were also observed at 50 and 162 kDa (Figure 2B).

Conglutinin was detected not only in the serum of adult animals, but also in colostrum and milk from cows and in foetal bovine serum. In Western blot profiles of conglutinin derived from colostrum and milk and from foetal bovine sera – both the commercial sera and those obtained from two calves immediately after birth – four dominant bands were observed with molecular masses of 153, 280, 338 and 387 kDa, and a few with lower optical density. In the case of the non-commercial foetal sera, a strong reaction also appeared at 79 kDa (Figure 3).

Goat and sheep serum lectins of similar antigenicity to bovine conglutinin

The lectin fractions obtained from the sera of different breeds of goats and sheep were incubated with antibodies specific for bovine conglutinin. In immunoblotting using monoclonal antibodies, five main bands characteristic of bovine serum conglutinin were observed in the profiles of non-reduced goat and sheep proteins. In all the breeds studied a reaction was evident at 153 kDa, and in some breeds also at 175, 280, 338 and 387 kDa (Figure 4). In the profiles of reduced goat and sheep serum lectins, weak reactions were observed with rabbit polyclonal antibodies at 45 and 48 kDa. In the case of Boar goat and Merinos sheep lectins, an additional third band appeared corresponding to a molecular mass of 41 kDa (Figure 5).

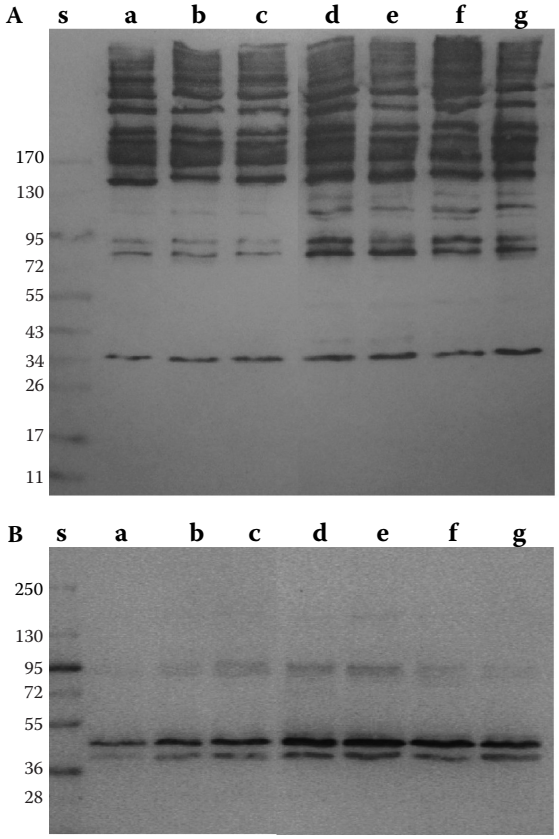

Figure 2. Western blotting of lectin fractions derived from the sera of different breeds of cattle. To isolate serum lectins, sera were incubated with mannan-agarose medium (Sigma) and lectins were eluted with TBS-EDTA buffer. The protein fractions obtained were loaded onto 3 to 20% polyacrylamide gel and after completion of electrophoresis transferred onto PVDV membrane. **A** = reactions of non-reduced conglutinin with monoclonal antibodies specific for bovine conglutinin (mab 236-01, Abcam); **B** = reactions of reduced conglutinin (treated with 50mM 2-mercaptoethanol) with anti-conglutinin rabbit serum; **a** = Polish Lowland cattle; **b** = Holstein-Friesian red and white; **c** = Holstein-Friesian black and white; **d** = Piemontese; **e** = Limousine; **f** = Aberden Angus; **g** = Charolaise; **s** = molecular weight standard (Fermentas)

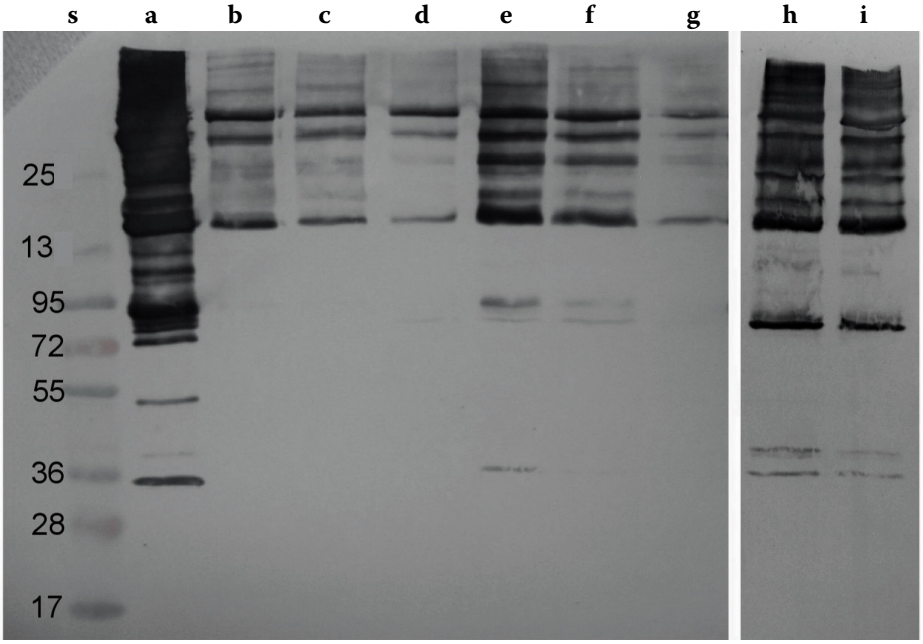

Figure 3. Western blotting of conglutinin derived from bovine colostrum, milk and foetal sera. To isolate conglutinin the material was incubated with mannan-agarose medium (Sigma) and lectins were eluted with TBS-EDTA buffer. The protein fractions obtained were loaded onto 3 to 20% polyacrylamide gel and after completion of electrophoresis transferred onto a PVDV membrane. Monoclonal anti-bovine conglutinin antibodies were used to detect non-reduced conglutinin (mab 263-01, Abcam). **a** = normal bovine serum (Polish Lowland cattle); **b** = colostrums; **c** = milk; **d** = FBS collected in USA (Sigma, F6178); **e** = FBS collected in Australia (Gibco, 10099-133); **f** = FBS collected in South America (HyClone, SV30160.02); **g** = FBS collected in EU-approved countries (Biochrom, S0113), **h** = FBS (calf 1); **i** = FBS (calf 2); **s** = molecular weight standard (Fermentas)

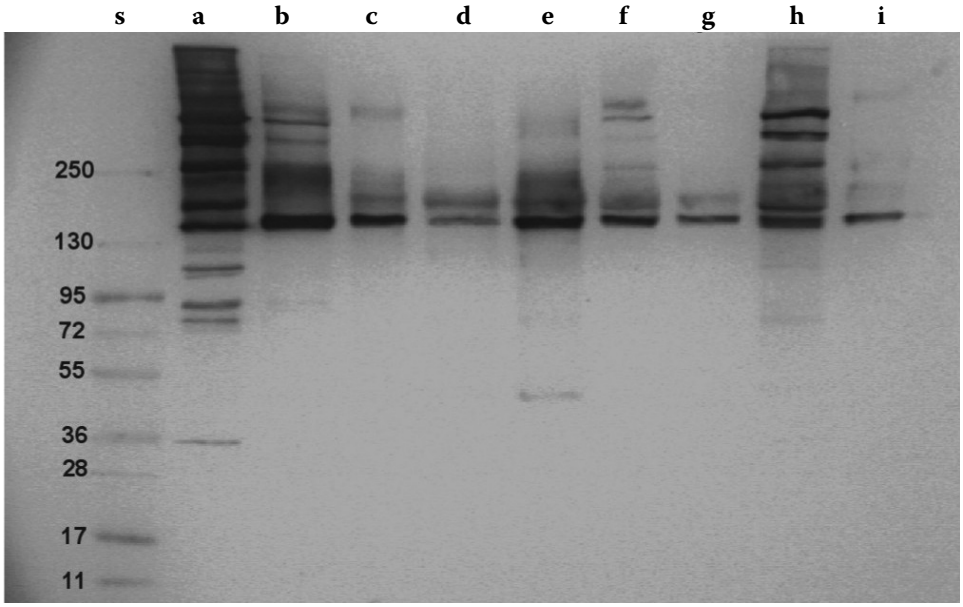

Figure 4. Western blotting of lectin fractions obtained by incubating sera of different breeds of goats and sheep with mannan-agarose medium (Sigma). Lectins were eluted with TBS-EDTA buffer, loaded onto 3 to 20% polyacrylamide gel and after completion of electrophoresis transferred onto a PVDV membrane. Monoclonal anti-bovine conglutinin antibodies (mab 263-01, Abcam) were used to detect non-reduced conglutinin. **a** = Polish Lowland cattle; **b** = Boer goat; **c** = Saanen goat; **d** = White Improved goat; **e** = Syrian goat; **f** = Polish Lowland sheep Uhruska variety; **g** = Suffolk; **h** = Merynos; **i** = synthetic prolific meat line sheep (BCP); **s** = molecular weight standard (Fermentas)

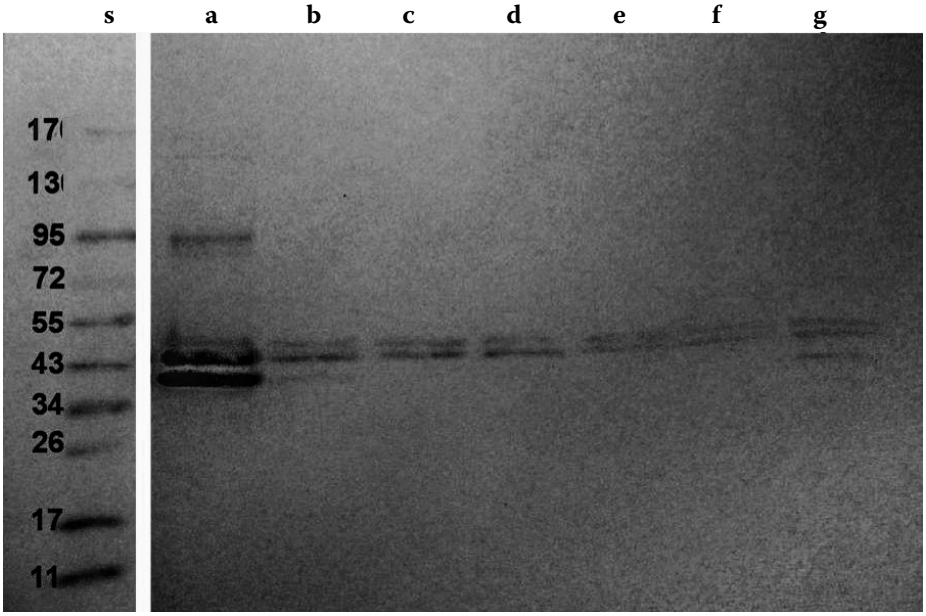

Figure 5. Western blotting of lectin fractions obtained by incubating sera of different breeds of goats and sheep with mannan-agarose medium (Sigma). Lectins were eluted with TBS-EDTA buffer, loaded onto 3 to 20% polyacrylamide gel and after completion of electrophoresis transferred onto a PVDV membrane. Rabbit anti-conglutinin serum was used to detect reduced conglutinin. **a** = Polish Lowland cattle; **b** = Boer goat; **c** = Saanen goat; **d** = White Improved goat; **e** = Polisk Lowland sheep Uhruska variety; **f** = Suffolk; **g** = Merynos; **s** = molecular weight standard (Fermentas)

Cross reactions of antibodies specific for bovine conglutinin with lectins of other species

In the next stage of the experiment, sera were used from humans and from the following animal species: European bison, deer, gnu antelope, llama, horse, boar, and swine. Reactions were observed in Western blotting between lectins isolated from the sera and antibodies specific for bovine conglutinin. The profile closest to that of bovine conglutinin, under both non-reduced and reduced conditions, was obtained in the case of the European bison and swine lectins. The non-reduced bison conglutinin was composed of 9 bands with molecular masses from 79 to 630 kDa, most of which correspond to bovine conglutinin fractions. The strongest reactions occurred with bands of 160 and 387 kDa. In the profile of non-reduced swine lectin three dominant bands were observed, with molecular masses of 153, 175 and 387 kDa, and weaker reactions at 34, 80, 233, 262 and 630 kDa (Figure 6A). The masses of these bands corresponded to bands comprising the protein profile of bovine conglutinin.

The reduced serum lectins derived from the animals studied (European bison, deer, gnu antelope, llama, horse, boar, and swine) reacted with polyclonal antibodies specific for bovine conglutinin. The immunoblot profiles were composed of one or two bands of low optical density appearing at about 40 kDa. Three bands were observed only in the profile of European bison lectin, appearing at masses corresponding to bovine conglutinin fractions (41, 47, 96 kDa). Two reactions occurred in the profile of swine lectin, at 39 and 44 kDa (Figure 6B).

DISCUSSION

The results of this study show that conglutinin occurs not only in the serum of adult cattle of various breeds, but also in the colostrum and milk of cows and in bovine foetal serum. Moreover, lectins which react with mono- and polyclonal antibodies specific for bovine conglutinin are present in the serum of other ruminants (bison, deer, gnu antelope) and non-ruminants (llama, horse, boar, pig), as well as that of humans. The basis for identification of bovine conglutinin and detection of proteins with similar antigenicity was reactions with monoclonal antibodies, which are more specific than polyclonal antibodies. However, because monoclonal antibodies only react with non-reduced conglutinin, rabbit antiserum was used to identify reduced conglutinin.

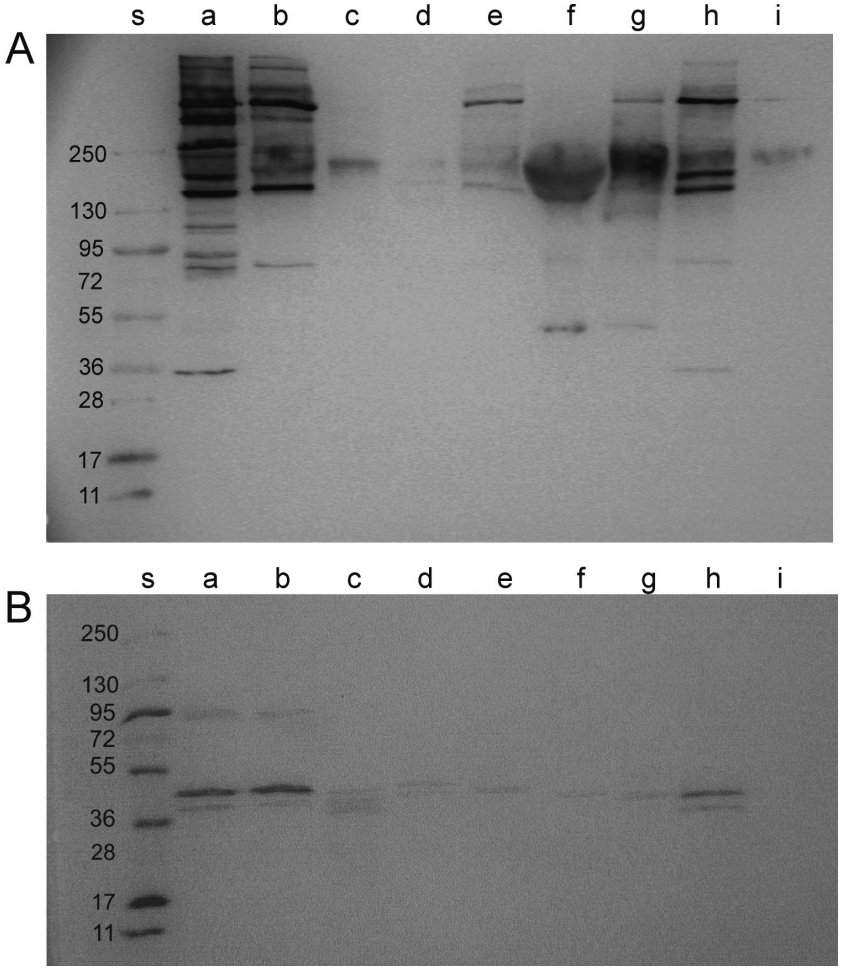

Figure 6. Western blotting of lectin fractions obtained from the sera of different ruminant and non-ruminant species. To isolate conglutinin sera were incubated with mannan-agarose medium (Sigma) and lectins were eluted with TBS-EDTA buffer. The protein fractions obtained were loaded onto 3 to 20% polyacrylamide gel and after completion of electrophoresis transferred onto a PVDV membrane. **A** = reactions of non-reduced conglutinin with monoclonal antibodies specific for bovine conglutinin (mab 236-01, Abcam); **B** = reactions of reduced conglutinin (treated with 50mM 2-mercaptoethanol) with anti-conglutinin rabbit serum; **a** = Polish Lowland cattle; **b** = European bison; **c** = deer; **d** = gnu antelope; **e** = llama; **f** = horse; **g** = boar; **h** = pig; **i** = human; **s** = molecular weight standard (Fermentas)

The Western blot profiles of non-reduced serum conglutinin from the dairy and meat breeds tested took the form of a ladder composed of many bands with molecular masses ranging from 34 to 630 kDa. The slight differences between the conglutinin protein profiles of different breeds, involving a few bands of very low optical density (39, 42, 51, 64, 107 kDa), were probably due to differences in the concentration of the protein applied to the gel; the material analysed was a mixture of serum lectins, so the concentration of conglutinin may have varied somewhat in different samples. The profile of reduced conglutinin consisted of three bands with molecular masses of 41, 47 and 96 kDa. In some cases weak reactions also appeared at 50 and 162 kDa. The results presented are consistent with the observations of other authors. According to Suzuki et al. (1997) reduced conglutinin takes the form of two main bands with masses of 42.7 and 45 kDa, and Holmskov et al. (1995) reported two bands of 40 kDa (minor band) and 44 kDa (major band). Andersen et al. (1992), in a profile of reduced conglutinin analysed in Western blotting using rabbit antiserum, observed one main band of 43 kDa (which in some experiments split into two bands) and weaker bands corresponding to molecular masses of 86 and 180–190 kDa (double band). In profiles of non-reduced conglutinin, Lu et al.

(1993), Holmskov et al. (1995) and Krogh-Meibom et al. (2004) distinguished several fractions with molecular masses above 120 kDa and one fraction with a low mass of about 35 kDa.

The presence of conglutinin in the colostrum and milk of cows results from its transport from serum to the mammary gland, as in the case of immunoglobulin. The secretion of conglutinin to the colostrum probably explains why its concentration in the serum of cows decreases at calving time. The conglutinin titre in whey obtained from colostrum and milk is generally low – less than five – while the titre in the serum of adult animals is usually between 80 and 320 (Ingram and Barnum, 1965). Low concentrations of conglutinin in colostrum, milk and bovine foetal serum were observed in this study as well.

The presence of conglutinin in bovine foetal serum indicates that the protein is synthesized during foetal life. Conglutinin, like immunoglobulin, cannot be transported through the placenta from the mother's blood to the foetus due to its high molecular mass. Ingram and Barnum (1965) reported that the serum of most precolostral calves does not exhibit conglutinating activity. Conglutinin does not appear in the serum of calves until after it has received colostrum; then its concentration gradually increases up to the 80^{th} week of life, when it reaches the level characteristic for adult cattle. Other authors have shown that precolostral calf serum exhibits conglutinating activity and contains approximately 1 to 3% of adult levels of conglutinin (Tlaskalova and Barta, 1968, Triglia and Linscott, 1980).

Monoclonal and polyclonal antibodies specific for bovine conglutinin react not only with bovine conglutinin but also with serum lectins of goats, sheep and swine. This is demonstrated by the occurrence of proteins in the serum with similar antigenicity to that of conglutinin. A study by Kakoma and Kinyanjui in 1974 confirmed that conglutinating activity is exhibited not only by serum from cattle but also from African ruminants, such as African buffalo, waterbuck, Uganda kob, Kenya kob, dik-dik, topi and Jackson's hartebeest, as well as from the dromedary. Sera from other ruminants, however, such as from moose and caribou, do not exhibit conglutinating activity (Mittal et al., 1982). According to Lachmann (1967), conglutinin also does not occur in the sera of sheep, mice, rats, rabbits, guinea pigs, horses, cats, dogs, pigs or humans. Tlaskalova and Barta (1968), however, confirmed the presence of a conglutinating factor in pig serum, both from adults and from precolostral piglets. These authors even suggested the term 'pig conglutinin'. Unlike in cattle, however, conglutinating activity in pig serum is not inhibited by adding zymosan or *N*-acetyl-*D*-glucosamine to the mixture of alexinated erythrocytes and serum. The present study's detection in sheep of a protein with similar antigenicity to that of conglutinin is confirmed by Lu et al. (1993), who used the Northern blot technique to detect mRNA for conglutinin in sheep liver. The signal obtained was faint compared to the RNA isolated from cattle liver, which indicates differences in the nucleotide sequences of the mRNAs detected (weak affinity of the probe for sheep mRNA), which translate into differences in the amino acid structure of cattle and sheep conglutinin. The presence of conglutinin in sheep is also indicated by a study by Jonas and Stankiewicz (1984), who found, in contrast to the results presented by Lachmann (1967), that the sera of some sheep exhibit conglutinating activity.

In the immunoblot reaction of a human non-reduced serum lectin fraction with monoclonal antibodies specific for bovine conglutinin, two bands were observed with low optical densities at 233 and 387 kDa. Under reduced conditions, however, no reaction was noted between the human protein and the rabbit antiserum specific for conglutinin. Thiel et al. (1987) detected a protein in human plasma with similar properties to those of bovine conglutinin. In the presence of calcium ions it showed affinity for IgG antibodies treated with complement and for zymosan. In immunoblotting it reacted with polyclonal antibodies specific for bovine conglutinin – non-reduced protein at 330 kDa, and reduced protein at 66 kDa (Baatrup et al., 1987).

In summary, conglutinin derived from the sera of various breeds of cattle takes the form of a ladder composed of many fractions with molecular masses ranging from 34 to 630 kDa, when analysed in Western blotting under non-reducing conditions. The reduced conglutinin profile takes the form of 3 main bands with masses of 41, 47 and 96 kDa. The presence of conglutinin in bovine foetal serum and in the colostrum and milk of cows indicates that this protein plays an important role in newborns. The detection of lectins with similar antigenicity to that of bovine conglutinin in the sera of other species, like goat, sheep and pigs, forms the basis for further research and suggests that conglutinin may even occur in non-ruminants.

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