Molecular characterization of the major capsid protein VP6 of bovine group B rotavirus and its use in seroepidemiology

Hiroshi Tsunemitsu,1 Mariko Kamiyama,1 Kenji Kawashima,1 Ken Katsuda,1 Mariko Kohmoto,1 Linda J. Saif,2 Tomotaro Shouji1 and Toshiyuki Onodera1

Correspondence
Hiroshi Tsunemitsu
/tsunemi@affrc.go.jp

1Shichinohe Research Unit, National Institute of Animal Health, Shichinohe, Aomori 039-2586, Japan
2Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH 44691-4096, USA

The major inner capsid protein (VP6) gene of the bovine group B rotavirus (GBR) Nemuro strain is 1269 nt in length and contains one open reading frame encoding 391 aa. Nucleotide and amino acid sequence identities of the Nemuro VP6 gene compared with the published corresponding human and rodent GBR genes were respectively 66–67 and 70–72 %, which are notably lower than those between human and rodent viruses (72–73 and 83–84 %, respectively). Overall identities of VP6 genes among GBRs were substantially lower than those among both group A rotaviruses (GARs) and group C rotaviruses (GCRs) derived from different species of mammals. These results demonstrate that bovine GBR is remarkably distinct from other GBRs and that GBRs from different species may have had a longer period of divergence than GARs and GCRs. Recombinant VP6 was generated with a baculovirus expression system and used for an ELISA to detect GBR antibodies. All 13 paired sera from adult cows with GBR-induced diarrhea showed antibody responses in the ELISA. In serological surveys of GBR infection using the ELISA, 47 % of cattle sera were positive for GBR antibodies, with a higher antibody prevalence in adults than in young cattle. In pigs, a high prevalence of GBR antibodies (97 %) was detected in sera from sows. These results suggest that GBR infection is common in cattle and pigs, notwithstanding the scarcity of reports of GBR detection in these species to date.

INTRODUCTION

Rotavirus infections are a major cause of viral diarrhoea in children and young animals (Kapikian et al., 2001). At present, rotaviruses are assigned to six groups (A–F) based on antigenic and genomic analysis (Bridger, 1994; Pedley et al., 1983; Saif, 1990). Each group possesses its own cross-reactive (group) antigens and shows similar double-stranded (ds) RNA electrophoretic-migration patterns, which vary among groups. The majority of group A rotaviruses (GARs) are differentiated based on the origin of the host species (such as human and bovine rotaviruses), although natural GAR cross-species infection and disease have been identified (Kapikian et al., 2001; Kojima et al., 1996; Nakagomi & Nakagomi, 2002; Taniguchi et al., 1994).

A similar distinction may exist in other rotavirus groups, although few studies have focused on non-GARs.

Group B rotaviruses (GBRs) cause diarrhoea in humans, pigs, cattle, lambs and rats (Bridger, 1994; Bridger et al., 1982; Mackow, 2002; Mebus et al., 1978; Saif, 1990; Theil et al., 1985). In humans, GBRs have been associated with large outbreaks of gastroenteritis, mainly in adults in China; however, outside China, there have been only a few reports of GBR detection (Ahmed et al., 2004; Hung et al., 1984; Kobayashi et al., 2001; Krishnan et al., 1999; Mackow, 2002). In pigs, GBRs have been detected in nursing- and weanling-pig diarrhoea (Bridger, 1994; Janke et al., 1990; Saif, 1990) and GBR infection in cattle has been reported in the USA, UK, India and Japan (Chang et al., 1997; Khurana & Pandey, 2001; Saif et al., 1991; Snodgrass et al., 1984; Tsunemitsu et al., 1999). Outbreaks of diarrhoea in dairy cows caused by bovine GBR have been observed in Japan (Hayashi et al., 2001; Tsunemitsu et al., 1999). However, reasons for the prevalence of GBR infection in cattle remain unclear.
because, with the exception of one porcine strain (Sanekata et al., 1996), serial propagation of GBRs in cell culture has been unsuccessful and immunological reagents for detecting GBR antigens and antibodies are available in only a few laboratories.

The major capsid protein of rotaviruses, VP6, is highly antigenic and immunogenic. VP6 is a major component of the virion, accounting for approximately 51% by weight of the virion in GARS (Mattion et al., 1994). Group-antigen epitopes are detected predominantly on VP6 in each rotavirus group (Kapikian et al., 2001; Mattion et al., 1994). In GARS, group antigens generally cause extensive cross-reactivity between strains in serological tests, e.g. immunofluorescence (IF) and enzyme-linked immunosorbent assays (ELISA). Therefore, several diagnostic assays directed against these antigens have been developed for GAR infection and are available for use with clinical samples from many species, at least among mammals. In contrast, most avian GARS react with only a limited set of broadly cross-reacting mammalian VP6-specific mAbs (Brüssow et al., 1992), suggesting the absence of certain epitopes of group antigens in avian GARS (Saif et al., 1994). These antigenic relationships correspond to the similarity in VP6 polypeptide sequences, with >87% amino acid identity within mammalian GARS and 70–75% amino acid identity between mammalian and avian GARS (Ito et al., 1995; Tang et al., 1997).

Information about the extent of VP6 variation in GBRs is limited. To date, GBR VP6 gene sequences have been determined only for human and rodent species (Chen et al., 1991; Eiden et al., 1992; Mackow, 2002). The extent of VP6 variation between human and rodent strains was greater than that reported for heterologous mammalian GARS, although recombinant VP6 from the rodent strain IDIR reacted with antisera against homologous and heterologous GBRs, including the human strain ADRV (Lindsay et al., 1993). On the basis of nucleic acid hybridization, Eiden & Allen (1992) indicated that the VP6 gene of the IDIR strain might be related more closely to that of the ADRV strain than to that of bovine GBR. There have been no further reports, however, on the genetic characterization of the VP6 gene of bovine GBR.

In this report, we determined the complete sequence of the VP6 gene of the bovine GBR Nemuro strain, derived from an adult cow with diarrhoea in Japan. To our knowledge, this is the first report on the GBR VP6 gene sequence from a domesticated animal. Sequence comparison with the cognate genes of human and rodent GBRs showed a remarkable diversity among GBRs, suggesting that, compared with GARS and group C rotaviruses (GCRs), GBRs derived from different mammalian species are more genetically divergent. We also generated recombinant VP6 protein with a baculovirus expression system and developed an ELISA using the expressed protein for the detection of GBR antibodies in cattle and pig sera. Our seroepidemiological study indicated the common occurrence of GBR infection in cattle and pigs and the necessity of clarifying its aetiological role in diarrhoea in these animals.

**METHODS**

**Virus and antisera.** The bovine GBR Nemuro strain, isolated from the faeces of adult cows with diarrhoea in Japan, was propagated in colostrum-deprived calves (Tsunemitsu et al., 1999). The virus was partially purified from faeces by ultracentrifugation through a 30% sucrose cushion in 0·1 M Tris-buffered saline [0·1 M Tris/HCl, 0·1 M NaCl (pH 7·5)] and dsRNA was extracted from the pellet with TRizol LS reagent (Invitrogen). Antiserum to bovine GBR (ATI strain) was prepared in a gnotobiotic calf. Briefly, a gnotobioc biofalt that had convalesced from an oral infection was given an intramuscular injection of virus-laden gnotobioc calf-intestinal contents combined with Freund’s complete adjuvant. Hyperimmune antiserum to bovine GAR and GCR were as described previously (Tsunemitsu et al., 1999).

**Serum specimens.** In total, 520 serum samples obtained from cattle and pigs were tested for antibodies for GBR. The 380 bovine serum samples included (i) 13 paired sera from adult cows with field GBR infections from two outbreaks in Japan (Hayashi et al., 2001; Tsunemitsu et al., 1999), (ii) four convalescent-phase sera from colostrum-deprived calves that were inoculated experimentally with bovine GBR Nemuro or GAR 22R (Matsumura et al., 2002), (iii) eight pre-exposure sera collected from colostrum-deprived calves and (iv) 342 sera from 33 farms of dairy cattle in Japan, aged between 4 months and 11 years. The 140 porcine serum samples included (i) 10 pre-exposure sera from gnotobioc pigs, (ii) 30 sera from a farm of pigs grouped by age from 1 to 6 months and (iii) 100 sera from nine farms of sows in Japan.

**Cloning and sequence analysis.** Synthetic primer 1 (5’-CCCG-TGCGCAATTTT-T3’-NH2) was ligated to the 3’ ends of the viral RNA as described by Lambden et al. (1992). cDNAs were produced by RT-PCR using primer 2 complementary to primer 1 and an oligonucleotide primer corresponding to either the plus sense of the 5’ end or the minus sense of the 3’ end of the ADRV GBR VP6 gene (Chen et al., 1991). The cDNAs were cloned into the pGEM-T vector (Promega) and sequenced by cycle sequencing with an automated DNA sequencer (ABI PRISM 310; Applied Biosystems). Confirmation of both terminal sequences was also provided by use of a 5’ rapid amplification of cDNA ends system (5’ RACE; Invitrogen). The full-length VP6 gene cDNA was produced by RT-PCR with 5’- and 3’-end primers designed with the determined terminal sequences, and the PCR product was also sequenced directly by cycle sequencing.

**Construction of baculovirus recombinant.** The full-length Nemuro VP6 gene cDNA was produced by RT-PCR with the oligonucleotides 5’-ACGAATTCAGTGCTTCCATCAGGGTG-3’ [primer sequence with a flanking EcoRI site (underlined) and the 5’ end of the VP6 gene of the Nemuro GBR] and the 5’-ATGGAATTCGCTC- TCTAGACAAACCGGTCTC-3’ [primer sequence with a flanking BamHI site (underlined) and the complement of the 3’ end of the VP6 gene of the Nemuro GBR]. The PCR product was digested with EcoRI and BamHI and cloned into the EcoRI–BamHI sites of the pVL1392 vector (BD PharMingen). Cotransfection of Spodoptera frugiperda (Sf9) cells with the recombinant pVL1392 and the Baculagold linearized baculovirus DNA (BD PharMingen) was performed by using Lipofectin (Invitrogen) in accordance with the manufacturer’s instructions. The expression of GBR antigens in Sf9 cells infected with the baculovirus recombinant was identified by indirect IF (Tsunemitsu et al., 1999) and Western blot assays.

**SDS-PAGE and Western blot assay.** Sf9 cells were infected with the baculovirus recombinant or wild-type (wt) Autographa californica multiple nuclear polyhedrosis virus (AcNPV) at an m.o.i. of 10 p.f.u. per cell and incubated for 3 days. Harvested cells were washed twice with PBS (pH 7·4). Cells were dissolved in SDS-PAGE
sample buffer [12 mM Tris/HCl (pH 6.8), 5% glycerol, 0.4% SDS, 10% β-mercaptoethanol and 0.02% bromophenol blue] and boiled for 10 min. Proteins were dissociated by SDS-PAGE analysis with 4% stacking and 10% separating gels. Proteins on the gel were stained with Coomassie blue or electroblotted onto a PVDF membrane (Bio-Rad). The membrane was blocked with 5% BLOTTO at 37°C for 1 h and washed five times with PBST. Finally, ABTS (0.05% Tween 20 in PBS) and incubated for 1 h and clarified by centrifugation at 10,000 g for 20 min. Each well received 100 μl of the test sera at 4°C overnight and washed five times with PBST. Next, plates were incubated at 37°C for 1 h and washed five times with PBST. Finally, ABTS [2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] substrate was added and A405 was recorded. The GBR antibody data were expressed as net A405 of samples >7SD above the mean value for sera from pre-exposure colostrum-deprived calves or from pre-exposure gnotobiotic pigs.

**RESULTS**

**Sequence analysis of VP6 gene of the bovine GBR Nemuro strain**

The full-length VP6 gene of the Nemuro GBR was 1269 nt in length and encoded one open reading frame encoding a polypeptide of 391 aa. The untranslated regions were 32 and 61 nt at the 5’ and 3’ ends, respectively. The gene was the same length as those of the human GBR strains (ADR, CAL-1, Bang373 and WH-1) and was 2 nt shorter than that of the rodent GBR IDIR strain (1271 nt). The lengths of the encoded amino acid sequences were the same for all GBR strains. The 5’ termini of these VP6 genes were conserved and started with the sequence 5’-GTT(A/T)T(A/T)AAAT(A/T)-. The 3’ termini of these genes were also conserved and ended with the sequence –(A/T)(A/T)AAAACCC-3’.

Comparison of the VP6 gene of the Nemuro strain with those from the human ADRV, CAL-1, Bang373 and WH-1 strains and the rodent IDIR strain indicated identities of 66–67% in nucleotide and 70–72% in deduced amino acid sequences, which were lower than those between human and rodent viruses (72–73 and 83–84%, respectively) (Table 1). The overall identities of VP6 genes among GBRs were

**Table 1. Nucleotide and deduced amino acid sequence identities of VP6 genes from group A, B and C rotaviruses**

<table>
<thead>
<tr>
<th>Rotavirus (serogroup/host/strain)</th>
<th>Nucleotide and amino acid sequence identity (%)†</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>KU</td>
</tr>
<tr>
<td>A/Hu/KU</td>
<td>76-6</td>
</tr>
<tr>
<td>A/Mu/EW</td>
<td>89-7</td>
</tr>
<tr>
<td>A/Bo/RF</td>
<td>91-4</td>
</tr>
<tr>
<td>B/Hu/ADRV</td>
<td>10-6</td>
</tr>
<tr>
<td>B/Hu/CAL-1</td>
<td>10-6</td>
</tr>
<tr>
<td>B/Hu/Bang373</td>
<td>10-6</td>
</tr>
<tr>
<td>B/Hu/WH-1</td>
<td>10-6</td>
</tr>
<tr>
<td>B/Mu/IDIR</td>
<td>10-6</td>
</tr>
<tr>
<td>B/Bo/Nemuro‡</td>
<td>10-1</td>
</tr>
<tr>
<td>C/Hu/Bristol</td>
<td>41-8</td>
</tr>
<tr>
<td>C/Bo/Shintoku</td>
<td>46-0</td>
</tr>
<tr>
<td>C/Po/Cowden</td>
<td>41-6</td>
</tr>
</tbody>
</table>

*Bo, Bovine; Hu, human; Mu, murine; Po, porcine.
†Sequence identity was determined by the CLUSTAL W method in the MegAlign program of Lasergene (DNASTAR). The upper right section is the nucleotide sequence identity and the bottom left is the deduced amino acid sequence identity. Identity within each serogroup is shown in bold.
‡The VP6 gene sequence was determined in the present study (GenBank accession no. AB106542).

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substantially lower than those among GARs and GCRs derived from different species of mammals (Table 1). Multiple alignments of the amino acid sequences of VP6 produced by using the MegAlign program (DNASTAR) indicated that the N-terminal regions of the VP6 were relatively conserved among GBRs; in contrast, however, no conserved regions were observed between GBR and GAR or GCR strains. Five of six cysteine residues in the Nemuro GBR occurred at positions identical to those in other GBR strains (aa 10, 194, 213, 278 and 337). Two (aa 194 and 337) of these were located almost identically in GAR (aa 197 and 331) and GCR (aa 188 and 336).

Expression of VP6 of the Nemuro GBR in Sf9 cells

On indirect IF testing, Sf9 cells infected with the baculovirus recombinant showed specific staining with antiserum to bovine GBR, but no reactivity was observed with antiserum to bovine GAR or GCR (data not shown). Sf9 cells infected with the baculovirus recombinant or AcNPV were lysed and analysed by SDS-PAGE. A 43 kDa protein in lysates from the baculovirus recombinant was observed, but not in control, AcNPV-infected cells (Fig. 1a), and this size was consistent with that deduced from the VP6 gene sequence. The 43 kDa protein reacted with antiserum to bovine GBR by Western blot assay (Fig. 1b). These results confirmed that the expressed protein was the GBR VP6.

Reactivity of ELISA for detecting GBR antibodies

The mean ± SD net $A_{405}$ of eight pre-exposure colostrum-deprived calves and 10 pre-exposure gnotobiotic pigs in the ELISA was $0.003 ± 0.025$ and $0.023 ± 0.023$, respectively. Therefore, a serum was considered GBR antibody-positive if the net $A_{405}$ was greater than 0.2.

Four convalescent-phase sera from newborn calves infected experimentally with bovine GBR (two calves) or GAR (two calves) and paired sera from 13 adult cows infected naturally with bovine GBR were used to evaluate the ELISA. Convalescent-phase calf sera directed against bovine GBR were positive for GBR antibodies until the serum was diluted 1:400–1:1600. In contrast, convalescent-phase calf sera against bovine GAR were negative in the ELISA (Fig. 2a). All paired sera from adult cows diluted 1:100 demonstrated increases in GBR antibodies in the ELISA (Fig. 2b).

![Fig. 1. SDS-PAGE (a) and Western blot assay (b) of baculovirus expression of the bovine GBR Nemuro VP6 gene. Western blot assay was conducted with antiserum to bovine GBR. Recombinant GBR VP6 and baculovirus polyhedrin are indicated by arrows on the left and right, respectively. Lanes: R, lysate from Sf9 cells infected with AcNPV–Nemuro VP6 recombinant baculovirus; A, lysate from Sf9 cells infected with wt AcNPV baculovirus; M, protein molecular mass marker.](image1)

![Fig. 2. Reactivity by ELISA of baculovirus-expressed Nemuro GBR VP6 for the detection of GBR antibodies. (a) Four convalescent-phase sera diluted serially from 1:100 to 1:25 600 from colostrum-deprived calves infected experimentally with bovine GBR Nemuro strain or GAR 22R strain. Symbols: ● and ■, convalescent-phase calf anti-GBR Nemuro sera; * and ○, convalescent-phase calf anti-GAR 22R sera. (b) Thirteen paired sera diluted 1:100 from adult cows with field GBR infection in acute and convalescent phases from two outbreaks in Japan. Values above the dotted line indicate positive results for GBR antibodies.](image2)
Table 2. Prevalence of GBR antibody in bovine sera grouped according to age, collected from 33 farms in Japan

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>No. tested</th>
<th>No. positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0*</td>
<td>52</td>
<td>6 (11.5)</td>
</tr>
<tr>
<td>1</td>
<td>49</td>
<td>11 (22.4)</td>
</tr>
<tr>
<td>2</td>
<td>43</td>
<td>19 (44.2)</td>
</tr>
<tr>
<td>3</td>
<td>39</td>
<td>16 (41.0)</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>21 (55.3)</td>
</tr>
<tr>
<td>5</td>
<td>34</td>
<td>23 (67.6)</td>
</tr>
<tr>
<td>6</td>
<td>36</td>
<td>30 (83.3)</td>
</tr>
<tr>
<td>7–11</td>
<td>51</td>
<td>36 (70.6)</td>
</tr>
<tr>
<td>Total</td>
<td>342</td>
<td>162 (47.4)</td>
</tr>
</tbody>
</table>

*Four to 11 months of age.

Prevalence of GBR antibodies in cattle and pigs

In total, 47 % of cattle sera from 29 of 33 farms were positive for GBR antibodies (Table 2). On comparison by age group, antibody-prevalence rates in calves (0–1 year of age) were 11–22 %, notably lower than the prevalence of 41–83 % in adult cows (Table 2). In pigs, 97 % of sow sera were positive for GBR antibodies. By age group at one farm, all pigs at 1 month of age were positive for GBR antibodies, which were assumed to be maternal antibodies. Antibody titres \( A_{405} \) values decreased at 2 months of age, then increased from 3 months (Fig. 3).

DISCUSSION

A marked sequence divergence of several genes between the human ADRV and rodent IDIR strains has been reported (Mackow, 2002). In recent sequence analyses of genes from additional human GBR strains (CAL-1, Bang373 and WH-1) (Ahmed et al., 2004; Kobayashi et al., 2001; Yang et al., 2004), genes from these human strains showed generally high sequence identities to that of ADRV, whereas identities with animal GBR strains were considerably lower. As for bovine GBR, we reported previously that the amino acid sequence identity of the bovine GBR Nemuro VP7 gene was high (96–97 %) and low (49–61 %) compared with the corresponding genes of bovine GBR strains detected in the USA and to those of the ADRV and IDIR strains, respectively (Tsunemitsu et al., 1999). In the present study, we demonstrated that the Nemuro VP6 gene showed low amino acid sequence identities to the corresponding human and rodent genes (70–72 %). These identity levels are similar to those between avian and mammalian GAR VP6s, which are differentiated into distinct clusters by phylogenetic analysis (Tso et al., 1995; Tang et al., 1997). These results suggest that GBRs from different host species are genetically distinguishable from each other and that bovine GBR might belong to a separate evolutionary lineage of GBRs. Confirmation of this, however, will require the accumulation and analysis of additional GBR field strains. Interspecies transmission and reassortment between strains from different animal species have yet to be observed in GARs.

The large divergence of GBR VP6 sequences from different species suggests that the GBRs of different species may have had a longer period of time to diverge from one another than have GARs and GCRs. Furthermore, VP6 sequence diversity might reflect previous observations of antigenic differences between GBRs in reactivity on immunoelectron microscopy and ELISA (Eiden et al., 1986; Nakata et al., 1986), despite of the presence of group antigens on VP6 (Eiden et al., 1994; Yolk et al., 1988). VP6 among GBRs from different species may have fewer epitopes that define group antigenicity than those of GARs and GCRs.

Comparisons of VP6 sequences between different serogroups confirmed that GAR and GCR VP6 are related more closely to each other than to GBR VP6. Antigenic cross-reactivity has never been observed between GBRs and GARs or GCRs (Saif, 1990), whereas we previously reported the presence of cross-reactive epitopes on VP6 between GARs and GCRs (Tsunemitsu et al., 1992).

Disulfide bonds have been implicated in the intramolecular tertiary structure of the GAR and GCR VP6 (Estes et al., 1987; Sabara et al., 1987; Tosser et al., 1992). All GBR VP6 proteins contain six cysteine residues, double the number found in GAR and GCR VP6 proteins (Mattion et al., 1994), and five cysteine residues are conserved within the GBR VP6. Two of these are located almost identically in GAR and GCR VP6, suggesting that these cysteine residues are likely to be involved in the tertiary forms of rotavirus VP6 functional domains.

The production of large amounts of recombinant Nemuro VP6 expressed in the infected Sf9 cells facilitated the development of an ELISA for GBR-antibody detection. The antibody ELISA in the present study was specific for GBRs and showed no reaction to convalescent-phase sera of calves infected experimentally with GAR. Further, the sensitivity...
of the assay is suitable for the investigation of bovine GBR infection because all paired sera from cows infected naturally with GBR showed an increase in GBR-antibody titres. In the present study, we were not able to examine the reactivity of the recombinant bovine VP6 with antisera against human and rodent GBRs.

There have been few reports of the antibody prevalence of GBR in cattle. No GBR antibody was found in cattle sera in China (Hung et al., 1985), whereas 20–71 % of cattle sera were positive for GBR antibodies in the UK (Bridge, 1987; Brown et al., 1987). The antibody prevalence in the present study was similar to that in the UK. The positive rate of GBR antibodies in sera from young cattle was lower than that in adult cattle. This result suggests that GBR infection in cattle is common in Japan, especially in adult cattle, supporting our recent reports on outbreaks of diarrhoea in adult cows with GBR infection (Hayashi et al., 2001; Tsunemitsu et al., 1999). Further, the difference in antibody prevalence among age groups in the present study may indicate that the risk of GBR exposure in cattle is age-related. Interestingly, similar findings have been reported for GBR and GCR infections in cattle (Hung et al., 1993; Steele & James, 1999). Further studies are needed to compare the sensitivity to, and pathogenicity of, bovine GBR between young and adult cattle.

We could not verify the sensitivity of the present ELISA against porcine GBR infection. However, this assay showed a very high prevalence of GBR antibodies in sera from adult pigs, suggesting that it may also be suitable for the investigation of porcine GBR infection. The prevalence of GBR antibodies in pigs varies among reports, with infection spread widely in the UK and Australia (antibody prevalence of 59–97 %), but apparently less widely in China and the USA (23–36 %) (Bridge & Brown, 1985; Brown et al., 1987; Hung et al., 1985; Oishi et al., 1993; Steele & James, 1999). Further studies are needed to compare the sensitivity to, and pathogenicity of, bovine GBR between young and adult cattle.

REFERENCES


