Bovine Ephemeral Fever in Taiwan (2001–2002)

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(Received 24 June 2004/Accepted 8 December 2004)

ABSTRACT. Bovine ephemeral fever (BEF), a vector-borne disease of cattle, is caused by the Ephemerovirus of the family Rhabdoviridae. In the past 40 years, Taiwan has had seven BEF epizootics, and we have previously reported the first five. This study summarizes the 2001 and 2002 epizootics; conducted case-control serologic studies on 10 herds involved in the 2001 epizootic; determined whether the recent BEF viruses have varied significantly; and discusses the relationship between epizootic patterns and possible variant BEF viruses. For mature cows that had received at least 2 doses of vaccine before the study, a negative correlation between the prevaccinated (the 3rd dose and after) serum neutralization antibody (SNA) titers and their postvaccinated peak rates was found. When prevaccinated SNA levels were at ≤ 32, their postvaccinated SNA levels increased significantly faster (P<0.01) than for those at ≥ 32. The glycoprotein gene of isolates from 1999, 2001, and 2002 had a 99.2–99.9% homology, without consistent amino acid variations in the neutralization sites. Phylogenetic analysis of Taiwanese isolates revealed 2 distinct clusters, the 1983–1989 and 1996–2002 isolates. Cross-neutralization tests confirmed the glycoprotein gene sequence analysis results. In conclusion, annual boosters at SNA levels > 32, at more than 2 doses, or at intervals shorter than 6 months are not advisable. The occurrence of frequent small epizootics implies the dominance of BEF virus over host immunity, but not a variant virus.

KEY WORDS: bovine ephemeral fever, outbreak, serology, vaccination.

Bovine ephemeral fever (BEF) is a vector-borne viral disease of cattle that spans tropical and subtropical zones of Asia, Australia and Africa [1, 3, 10, 12]. The virus is transmitted via insect vectors, mosquitoes and Culicoides biting midges. Culicoides oxystoma and C. nipponensis are implicated, but have not yet been linked to BEF epizootics, in cattle herds of southern Taiwan [8]. The causative ephemero virus is bullet-shaped and consists of a minus sense, single-stranded RNA genome; five structural proteins, including one surface glycoprotein (G); and no non-structural proteins. The surface glycoprotein induces the production of a protective neutralizing antibody [5, 15, 16]. The disease is characterized clinically by the sudden onset of fever, stiffness, lameness, nasal and ocular discharges, depression, cessation of rumination, and constipation. The pathogenesis of BEF is complex [11, 14, 20], although it seems clear that host inflammatory responses, mediated by the release of cytokines, are involved in the expression of the disease [7, 12, 13].

In the past 40 years, Taiwan has had 7 epizootics (Table 1), three of which were focal outbreaks occurring in the past 4 years. These occurred despite the regimen of vaccination with inactivated virus begun in 1984 [2, 4]. We have previously reported the results of the first 5 epizootics and related studies [20]. This study summarizes the epizootics in 2001 and 2002; conducted case-control serologic studies on 10 herds affected by the 2001 epizootic; and determined, through nucleotide sequencing of the surface glycoprotein and cross-neutralization among different isolates, whether the recent viruses varied from previous ones. The relationship between epizootic patterns, host (population) immunity, and possible emergence of a variant BEF virus are also discussed.

MATERIALS AND METHODS

Sera sampling: In 2002, monthly surveys were conducted for serum neutralizing antibody (SNA). Samples were obtained from newborn calves (< 6 months old) and 1–10 year-old cows, primarily Holstein, raised in 10 herds affected by the 2001 epizootic (Table 1). Samples from newborn calves were monitored for the decay of the colostral antibody, the primary response to the 1st dose of the vaccine, and population immunity (Fig. 1). The SNA levels of cows older than 1-year-old were monitored as references for population immunity and the efficiency of the booster vaccination (Fig. 1 and Table 2). The vaccination policy was initiated in 1984. The 1st dose, a formaldehyde-inactivated virus, is given at 6 months of age, with boosters every 6 months. The annual booster is administered with the first dose in January or February and the second dose in June or July.

Serum neutralization antibody test and cross-neutralization experiments: The virus used was the TN-1999 strain [20], isolated from buffy coat cells and further adapted to baby hamster kidney (BHK-21 (C-13)) cells. Sera, inactivated at 56°C for 30 min, were 2-fold serially diluted with
serum-free minimal essential medium (MEM) to a maximum of 256-fold, the detection limit of this assay. Viral fluid (50 µl) containing 100 TCID_50 was mixed with 50 µl of diluted serum and reacted at 4°C overnight before the suspension of BHK-21 cells was added. The cytopathic effect (CPE) was observed 2 days later. The SNA titer was the reciprocal of the highest dilution in which at least 1 of the 2 wells exhibited inhibition of CPE. The time it took for a cow to increase its SNA titer at least 2-fold after vaccination was recorded, expressed as folds titer increase per month, and used to analyze the relationship between prevaccinated SNA titers and postvaccinated SNA peaking rates (Fig. 2). Since the detection limit was 1:256, the highest prevaccinated SNA titers incorporated in the analysis was 128 (i.e. 256 ÷ 2).

Table 1. Epizootics of BEF in Taiwan (1967–2002)

<table>
<thead>
<tr>
<th>Year</th>
<th>Disease Incidence (%)</th>
<th>Case Fatality (%)</th>
<th>Change of Epizootic Patterns</th>
<th>Number of Virus Isolates</th>
<th>Month of First Outbreak</th>
<th>District of First Outbreak</th>
</tr>
</thead>
<tbody>
<tr>
<td>1967</td>
<td>26.6 (1183/4441)</td>
<td>5.2 (62/1183)</td>
<td>Epidemic</td>
<td>None</td>
<td>Late March</td>
<td>Kaoshiung (Southern Taiwan)</td>
</tr>
<tr>
<td>1983–84</td>
<td>20.1 (5650/28117)</td>
<td>6.0 (340/5650)</td>
<td>Epidemic</td>
<td>4</td>
<td>October</td>
<td>Chiayi (Southern Taiwan)</td>
</tr>
<tr>
<td>1989–90</td>
<td>14.5 (4216/29157)</td>
<td>5.0 (210/4216)</td>
<td>Epidemic</td>
<td>11</td>
<td>April</td>
<td>Chiayi (Southern Taiwan)</td>
</tr>
<tr>
<td>1996</td>
<td>13.6 (14973/110247)</td>
<td>11.3 (1685/14973)</td>
<td>Epidemic</td>
<td>8</td>
<td>August</td>
<td>Hsinchu City (Central Taiwan)</td>
</tr>
<tr>
<td>1999</td>
<td>5.6 (538/9593)</td>
<td>21.9 (118/538)</td>
<td>Focal Outbreak</td>
<td>&gt; 2</td>
<td>October</td>
<td>Kaoshiung (Southern Taiwan)</td>
</tr>
<tr>
<td>2001</td>
<td>7.4 (1825/24816)</td>
<td>9.7 (177/1825)</td>
<td>Multi-Focal</td>
<td>11</td>
<td>October</td>
<td>Tainan (Southern Taiwan)</td>
</tr>
<tr>
<td>2002</td>
<td>15.0 (221/1476)</td>
<td>50.0 (111/221)</td>
<td>Focal Outbreak</td>
<td>&gt; 1</td>
<td>Junec)</td>
<td>Hualien (Eastern Taiwan)</td>
</tr>
</tbody>
</table>

a) See also reference 20.
b) The number of cows affected/total number of cows in the affected districts.
c) Includes the number of deaths and culls.
d) The number culled was 75. Four culls and 36 deaths were probably due to complication from bovine viral diarrhea based on gross intestinal pathology and bloody stool, although the disease has not been officially documented in Taiwan. The other 71 culls were mostly from recumbence due to arthritis and coma.
e) Later there was an outbreak in September involving few animals and no deaths in the herds also involved in the June outbreak.

Table 2. Significant SNA titers increase after annual 2-dose booster vaccination

<table>
<thead>
<tr>
<th>Populationa)</th>
<th>Titer (log_2) of Pre-B1b)</th>
<th>Titer (log_2) of B1-B2b)</th>
<th>Titer (log_2) of After-B2b)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N)c)</td>
<td>(N)</td>
<td>(N)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.89 ± 2.59d)</td>
<td>6.35 ± 1.55d)</td>
<td>6.81 ± 2.30d)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(170)</td>
<td>(755)</td>
<td>(1195)</td>
<td></td>
</tr>
<tr>
<td>Mature cows</td>
<td>5.39 ± 2.31d)</td>
<td>6.97 ± 1.24d)</td>
<td>7.22 ± 1.62d)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(140)</td>
<td>(602)</td>
<td>(959)</td>
<td></td>
</tr>
<tr>
<td>Immature cows</td>
<td>2.60 ± 2.62d)</td>
<td>3.89 ± 2.29d)</td>
<td>5.14 ± 2.86d)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(78)</td>
<td>(595)</td>
<td>(1212)</td>
<td></td>
</tr>
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</table>

a) Mature cows are those > 1 year old that had received at least two doses of basic vaccination by January 2002, the beginning of the study. Immature cows are those < 6 months old, which may or may not have received a vaccination before the study. Total is the combination of both mature and immature cows.
b) Annual 1st dose booster was generally done in January-February and 2nd dose in June-July.
Pre-B1=mean SNA titer ± standard deviation before 1st dose booster; B1-B2=mean SNA titer ± standard deviation after 1st dose and before 2nd dose, spanning several months; After-B2=mean SNA titer ± standard deviation after 2nd dose booster, also spanning several months until December 2002, the end of the study.
c) N = sample size.
d) Indicates significant difference (P<0.01) among the SNA titers on each row.
with an equal volume of incomplete Freund’s adjuvant, and this regimen was used as a booster a further 3 times before the serum was harvested. Each set of serum samples was then cross-neutralized with each virus using the method described above, except that the reaction was at 37°C for 1 hr.

**Analysis of glycoprotein gene sequence:** To determine whether the 2001 outbreak was caused by a BEF virus similar to or different from that of the 1999 outbreak, the glycoprotein genes of 11 isolates were sequenced and then compared to those previously isolated and sequenced. The method of virus isolation and the history of the other Taiwanese isolates identified by the year and district of isolation are summarized in Table 1 and are as reported before [20]. Each virus was purified by limited dilution, its RNA extracted, reverse transcribed [19], and amplified by PCR [20], using 4 pairs of primers (1F and 480R, 407F and 880R, 761F and 1581R, and 1322F and 1872R), yielding 473–820 bp of products. The PCR products were gel purified (Amerham Pharmacia Biotech Inc., NJ, U.S.A.) for nucleotide sequencing. Primers were designed according to the published sequences [17, 20], where 1F was 5’ATGTTCAAGGTCTTATAATTACC3’ (1 to 24), and 480R was 5’GCTTGGGGGTATTAGGA3’ (464 to 480); 407F was 5’GGAATACGGAGATGAATCAA3’ (407 to 426), and 880R was 5’ATTCTGTTCTATCTGTGTGC3’ (861 to 880); 761F was 5’TGAGGATGGAGAATGGTG3’ (761 to 780); 1581R was 5’TACAACAGCAGATAAAAC3’ (1564 to 1581); and 1322F was 5’AAATGGAATGATCTTTGTTT3’ (1322 to 1341), and 1872R was 5’TTATGATCAAAGAATCTGTCATCACC3’ (1846 to 1872). Nucleotide sequencing was

<table>
<thead>
<tr>
<th>Polyclonal rabbit sera raised against</th>
<th>Neutralization titer against BEF virus strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine-1983</td>
<td>431 [b]</td>
</tr>
<tr>
<td>TN-1996 [a]</td>
<td>8</td>
</tr>
<tr>
<td>TN-1999</td>
<td>4</td>
</tr>
<tr>
<td>TN-2001</td>
<td>19</td>
</tr>
</tbody>
</table>

Table 3. Cross-neutralization of rabbit sera raised against various BEF viruses

[a] Glycoprotein gene sequence was similar to that of TC-1996 (Fig. 3).
[b] The titers expressed are geometric mean titers calculated from 4 wells of two serum samples.

Fig. 1. Population serum neutralizing antibody (SNA) profile in 10 herds that experienced the 2001 epizootic and were studied in the year 2002. The ordinate indicates the mean titer using the product(s) of log2 of the SNA titer of each animal in the target population as raw data for analysis. Vertical lines indicate one standard deviation. The abscissa indicates the month in which the sera were tested. Open arrows indicate the times of booster vaccinations in January-February and June-July. Mature cows are those > 1 year old that had received at least two doses of basic vaccination by January 2002, the beginning of the study. Immature cows are those < 6 months old, which may or may not have received vaccinations prior to the study.
performed using an automated ABI PRISM 373 DNA Sequencer (Perkin-Elmer, CA, U.S.A.). The G gene of the various BEF viruses was analyzed utilizing the Clustal method of the MegAlign-expert sequence analysis software (DNASTAR Inc. WI, U.S.A.).

Statistical analysis: All data were analyzed using the SAS-PC System® version 8.1 for Windows (SAS Institute, NC, U.S.A.), with one-way analysis of variance (Table 2) and chi-square analysis (Fig. 2) included to test the significance at $P<0.05$.

RESULTS

The 2001 epizootic was a multifocal outbreak occurring in Tainan County in southern Taiwan, the same region that experienced the 1999 epizootic (Table 1). The incidence was similar to the case in 1999, but the case fatality rate was lower by half. The 2002 epizootic was a focal outbreak occurring in a geographically isolated region of Hualien County in eastern Taiwan, an area separated from the rest of Taiwan by the Central Mountain Range. In that region,
SNA levels were at 1–32 versus 64–128. When prevaccinated SNA levels were at >32, their postvaccinated SNA levels peaked more slowly, with most increasing 1–6 folds per month. When prevaccinated SNA levels were at ≤32, their postvaccinated SNA levels peaked significantly faster, with most increasing 8–128 folds per month. When prevaccinated SNA levels were at >32, their postvaccinated SNA levels peaked more slowly, with most increasing 1–6 folds per month. Significant differences (P<0.01) were found between the two groups, with either prevaccinated levels at 1–16 versus 32–128 or prevaccinated levels at 1–32 versus 64–128.

Attempts to analyze the relationship between the prevaccinated SNA levels, derived from the colostrum, and their postvaccination (the 1st dose conducted at 6 months old) peaking rates yielded inconclusive results. Because the 2001 epizootic occurred in a region also affected by the 1999 epizootic, one concern was whether the 2001 epizootic was caused by a variant BEF virus or one similar to that from 1999. Glycoprotein gene sequences of 11 isolates from the 2001 epizootic were determined, and there were no consistent variations in the amino acids of any neutralization sites compared to those of the 1999 virus (data not shown). Results also showed a 99.2–99.9% nucleotide and amino acid homology among the 1999, 2001, and 2002 isolates (GenBank accession numbers are AF208840, AY062166, and AY768942, respectively). Phylogenetic analysis also placed the 1996–2002 Taiwanese isolates in a distinct cluster different from the 1983–1989 Taiwanese isolates, from the Japanese strains, and from the Australian BB7721 strain (Fig. 3); and showed that the 2001 and 2002 viruses were closest to or likely derived from the 1999 virus. Cross-neutralization tests were conducted to see if the structure information, as revealed by glycoprotein gene sequences, matched the functional information as revealed by serum neutralization (Table 3). The results indicate that sera raised against various 1983–2001 BEF viruses yielded higher SNA titers when reacted with viruses of the same genomic cluster (Fig. 3), and yielded lower SNA titers when reacted with BEF virus of a different genomic cluster, and vice versa.

**DISCUSSION**

In successive Taiwanese epizootics, the incidence and the scale (number of cows, herds, and districts affected) decreased, while the frequency increased, as evidenced by shorter epizootic intervals between 1999 and 2002 (Table 1), raising suspicions that a variant BEF virus, judged by alterations in neutralization sites [18], had emerged. Yet the sequence data (Fig. 3) laid to rest these suspicions. However, 0.1–0.8 % heterogeneity among the 1999, 2001, and 2002 isolates was present throughout the backbone of the glycoprotein gene, suggesting that the 1999 BEF virus was slowly evolving. These BEF epizootics can be viewed as the final results of three-way competition among the epidemiology triangle: the existing BEF viruses, the environment, and the host. In this competition, the advantage went to the virus and the environment, in which factors such as temperature, humidity, sanitation, and herd management favor the proliferation of insect vectors and the spread of the BEF virus. Alternatively, host factors such as the health of the animals and population immunity may pose a selection pressure on the virus. Assuming there is only a single dominant BEF virus in a district, and that the population immunity remains advantageous for a period of time, the BEF virus would be forced to mutate. However, the frequency of epizootics in recent years (Table 1) suggests that the BEF virus had the advantage, and thus there was no need for the virus to mutate. This notion is supported by the glycoprotein gene sequence (Fig. 3) and cross-neutralization (Table 3) data showing that the 2001 BEF virus was similar to that of 1999. Conceivably, nurture of a variant BEF virus with mutated neutralization sites, may require a few years of quiescence (such as between 1967 and 1983; between 1989 and 1996, Table 1) when population immunity remains effective, thus forcing the virus to mutate. In this sense, with regard to the possible emergence of a variant BEF virus, the absence of epizootics could be indicative of trouble to come. If this concept is correct, an acute large-scale (incidence) sweeping epidemic, following a few years of quiescence, would indicate signifi-
cant changes in neutralization sites, while frequent smaller-scale outbreaks would indicate an absence of significant changes or, alternatively, indicate that the virus moves among herds, attacking those individual cows which have insufficient immunity against the BEF virus. The case fatality rate of an epizootic, however, may not be used as an indicator of the presence of a variant virus, as it may be influenced by the health status of the affected animals, the efficacy of the treatments, and the measures adopted. However, the presence of a variant BEF virus with high virulence properties is always possible whenever a large-scale epidemic occurs.

The SNA levels of the newborn calves decayed to a minimum by 4 months of age, suggesting that it is more appropriate, in the field, to conduct the primary vaccination at or before 4 months of age, instead of at 6 months of age, as current policy stipulates. However, this may create problems in the synchronization of vaccination schedules, as mature cows are boostered at 6-month intervals.

The negative correlation between prevaccinated SNA levels and their postvaccinated peaking rates (Fig. 2) implies that boosters given at SNA levels > 32, annual boosters of more than 2 doses, or at intervals shorter than 6 months, are ineffective, most likely because the immune system is saturated. Steady increases of postvaccinated SNA levels (Table 2) also suggest that the vaccine currently used and vaccination policy are, in a sense, reasonably effective.

Exactly which SNA titer provides protection is debatable. In routine practice, we arbitrarily consider 32 as protective [20]; however, there were instances in which animals contracted disease when their SNA levels were at > 32–64 (data not shown), and also instances when low SNA levels provided protection [6]. Data in Fig. 2 indicate that an SNA titer of 32 is the level at which a booster or even natural infection may induce a variety of peaking rates, further supporting the supposition that 32 is in fact the titer of choice for routine practice.

We conclude that 6 months of age for giving the 1st dose of vaccination to newborn calves may be too late in the field; an annual booster at prevaccinated SNA levels > 32, at more than 2 doses, or at intervals shorter than 6 months, is not advisable; and a high frequency of small-scale epizootics implies a dominance of BEF virus over host immunity or the virus moving among herds; thus, no observable variation in its neutralization sites is expected.

ACKNOWLEDGMENTS. The authors thank Dr. Y. P. HUNG for technical assistance. This work was supported by grants (NTU grant number 93A-2027, 93A-1121 (93–1.4-ID-01(11)), 93A-2098 from the Council of Agriculture, TAIWAN, R.O.C.

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