An Epidemic of Parapoxvirus Infection among Cattle: Isolation and Antibody Survey

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ABSTRACT. A disease characterized by papules, nodules, vesicles and, rarely, pustules and ulcers on teats was seen among cattle on a farm in Chiba Prefecture, Japan. A virus was isolated by inoculation of fetal bovine lung cells from a vesicle on a teat of an infected cow. The virus was subsequently passed in fetal bovine lung and muscle cells in which it produced complete cytopathic changes. The virus was identified by physicochemical examinations and electronmicroscopic observation as a parapoxvirus. A seroepidemiological survey was performed on antibody to the isolated virus by the agar gel immunodiffusion test. The isolated virus formed a precipitation line which cross reacted with other parapoxviruses isolated previously in Japan. The positive rate was more than 50% among cattle in the Kantō district. The positive rate increased with age. It was suggested that parapoxvirus infection might have already been prevalent among cattle in Japan.—KEY WORDS: cattle, dermatitis, mammilitis, papula, parapoxvirus.


Recently a disease characterized by papules, nodules, vesicles and pustules, which sometimes progressed to ulcers on teats has been seen among cattle in several areas of Japan. During the winter of 1996 to 1997, such a disease was seen on a farm in Chiba Prefecture. Epidemiological observation suggested that the disease was caused by viral infection, and clinical symptoms suggested the disease was mammilitis caused by bovine herpesvirus type 2 [18], papillomatisis due to bovine papillomavirus [14] or papular dermatitis induced by one of the parapoxviruses of ruminants [8–10]. We have tried to collect lesion materials for isolation of the virus from affected cattle in the field, and a virus was isolated. Virological investigations were carried out to identify the isolated virus. This paper presents clinical, virological and serological studies of the natural disease in cattle and the isolated virus.

MATERIALS AND METHODS

Virus isolation: Materials for virus isolation were collected by biopsy from vesicles on teats and peripheral blood of cattle with clinical symptoms. Lesion material was minced and mixed with Eagle’s MEM and centrifuged for 30 min at 2,500 g. The supernatant was inoculated onto fetal bovine lung (FBL) cells and the inoculated cultures were observed for at least 12 days after inoculation. Peripheral blood leukocytes were separated from blood after lysis of erythrocytes by mixing with two volumes of 0.83% NH4Cl solution, washed 3 times in PBS, and inoculated onto FBL cells at final concentration of 1 x 10^6/ml. The inoculated cells were washed twice with Eagle’s MEM after 48 hr and fresh medium was added. The cells were cultivated again for at least 12 days. The inoculated cell cultures were freeze-thawed three times and inoculated onto fresh FBL cells if a cytopathic effect (CPE) was not observed. The blind passage was repeated twice.

Control viruses: Four strains of parapoxvirus isolated previously in Japan and vaccinia virus were used as controls for serological tests. The Aomori and Iwate strains were isolated from a cow with bovine papular stomatitis and a sheep with contagious pustular dermatitis, respectively, and preserved in the National Institute of Animal Health [7]. The Ishikawa-B strain and the Ishikawa-S strain were isolated from a cow and a Japanese serow with papular stomatitis, respectively, and kindly supplied by the Nanbu Livestock Hygiene Service Center in Ishikawa Prefecture. Vaccinia virus (LC16mO strain), bovine herpesvirus type 1 (Los Angeles strain) and bovine viral diarrhea virus (Nose strain) were preserved in the National Institute of Animal Health [4, 5, 15]. All viruses were propagated in fetal bovine muscle (FBM) cells.

Cell cultures: FBL cell cultures and FBM cell cultures were prepared by an original tissue-culture method. FBL cells were used within 5 passages for virus isolation and FBM cells were within 20 passages for physicochemical examinations of the isolated virus and viral antigen preparation. MDBK cells were employed for antigen preparation of the isolated virus for the agarose gel immunodiffusion (AGID) test. These cells were cultivated at 37°C in Eagle’s MEM containing 5% fetal calf serum and 0.3% tryptose phosphate broth.

Electron microscopic observation: Culture fluids collected from FBM cells infected with the isolated virus and induced CPE were centrifuged for 30 min at 10,000 g. The supernatant was then centrifuged for 2 hr at 110,000 g.
The sediment was resuspended in distilled water and prepared by negative staining with 2% phosphotungstic acid (pH6.5) for examination by electron microscope (JEOL-JEM 1010, JEOL Co., Ltd., Tokyo, Japan).

Characterization of the isolated virus: The type of nucleic acid of the isolate was determined indirectly by means of 5-

iodo-2'-deoxyuridine (IUDR). The virus yields in the media

with and without IUDR (10^-4M) were compared. Bovine

herpesvirus type 1 and bovine viral diarrhea virus were used

as controls for DNA- and RNA-type viruses, respectively.

The culture fluids were collected at 24 hr and 48 hr after

inoculation with 10^3 TCID_{50}/0.1 ml of virus and the

infectivity was determined.

The size of the isolate was determined by filtration. Virus

susensions were passed through Millipore filters and the

filtrates were tiritated for virus infectivity.

Either and chloroform sensitivities were determined by

mixing 4 volumes of virus susensions and one volume of the

solvent. The mixture was held for 18 hr at 4°C with

occasional vigorous shaking. The solvent was removed by

centrifugation and evaporation.

Heat sensitivity was determined by keeping the viral fluid

in a water bath at 50°C for 30 min and infectivity before

and after the heating was compared.

Serological test: FBM and MBDK cells infected with the

isolated virus were used as antigens for the AGID test. The

other viruses used as controls for the serological tests were

propagated in FBM, and infected FBM were used as

antigens for the AGID test. The infected cells were detached

from the culture bottle by trypsinization or with a rubber

policeman, collected in a centrifugation bottle, and washed

3 times in PBS by low-speed centrifugation. The

centrifuged cell pellet was suspended in a small volume of

PBS, sonicated and used as the antigen for AGID tests. The

AGID tests were performed with minor modifications of

the method reported by Kono et al. [6]. The wells were 5 mm

in diameter, and six circumferential wells were placed at a

distance of 3 mm from the central well. The central well

was usually filled with the antigen and the other wells were

filled with undiluted serum samples. The gel diffusion plate

was allowed to stand at room temperature for 3 days and

precipitation lines were observed.

Control antiserum for the AGID test was found from

serum samples collected from cows in the field. It yielded

a single dense precipitation line that was specific for the

isolated virus, as demonstrated by a reaction of identity

with a post serum from a cow at 30 days after experimental

infection with the virus by the AGID test. The sample was

regarded as positive for the reaction when a precipitation

line was formed and continuously joined with the control

positive line formed between the antigen well and control

antiserum well. If a precipitation line was not formed but

the control line curved slightly toward the inside of the test

serum well, the sample was regarded as weak positive for

the reaction and classified as antibody positive serum.

Serum samples: Serum samples were collected from three

prefectures in the Kanto district of Japan. In principle,

more than 50 samples were collected randomly from cattle

on several farms in each prefecture. Swine sera were

collected from several farms and supplied by the Chiba

Prefectural Institute of Animal Health. Horse sera were

collected randomly from racehorses in the Miho Training

Center in Ibaraki Prefecture and supplied by the Epizootic

Research Station, Equine Research Institute of the Japan

Racehorse Association. Sheep sera were collected from

several farms in Hokkaido Prefecture and supplied by the

Hokkaido Branch Station of the National Institute of

Animal Health.

RESULTS

Clinical and epidemiological features: A 14-month-old

Holstein, was recognized to have an ulcer on a teat by a

veterinarian belong to the Hokubu Livestock Hygiene

Service Station in Chiba Prefecture. The cow was born and

grew up on this farm where 44 dairy cattle were kept. About

one week later, small round or oval shaped white papules or

nodules were observed on the teats of 30 cattle on the farm

and some of them developed pustules, vesicles or ulcers.

The disease was mild, with neither an apparent increase in

fever or a change in appetite, and pain was not noticeable.

Virus isolation: Specimens from cattle with clinical

symptoms produced no CPE in FBL cell cultures during the

12-day observation period, but one FBL cell culture

inoculated with homogenized vesicles showed signs of CPE

at 5 days after the first blind passage. After passage three

in FBL cell cultures, the CPE was more obvious and was

apparent within 48 hr. Inclusion bodies were found in

cytoplasm and infected cells detached from the glass. The

isolated virus was named the Chiba strain and used for

further studies. No virus was isolated from peripheral blood

leukocytes.

Electron microscopic findings: The typical parapoxvirus

particles were identified in the concentrated culture fluids

from infected FBM cells. The virus particles were ovoid,

>290 x 160 nm in size, with a surface filament (Fig. 1).

Characterization of the isolated virus: Propagation of the

Chiba strain and bovine herpesvirus type 1 was definitely

suppressed in the cells with IUDR, whereas there was no

significant difference in BVDV replication in the cells with

or without IUDR. These results showed that the Chiba

strain contained DNA rather than RNA. The Chiba strain

was inactivated less than one thousandth by treatment with

ether and completely with chloroform. Most of the

viruses passed through a Millipore filter with a pore size of

450 nm and some passed through 200 nm pores, but they

failed to pass through a filter with a pore size of 100 nm.

The virus caused intracytoplasmic inclusions, which were

clearly seen on hematoxylin and eosin staining.

Antigenic relationships among the Chiba strain and

previously isolated strains: As the properties of the Chiba

strain indicated that it was likely to be a parapoxvirus, the

antigenicity was compared by the AGID test with various

strains of parapoxviruses. The precipitation line formed
between the control antiserum and the Chiba strain connected with the precipitation line formed between the antiserum and the parapoxviruses strains (Fig. 2). These tests confirmed that the Chiba strain was closely related to the Aomori, Iwate, Ishikawa-B and Ishikawa-S strains but not to vaccinia virus. No precipitation line was formed by vaccinia virus propagated in FBM cells.

Distribution of antibody-positive cattle in the field: When 219 sera were collected from cattle in three prefectures in the Kanto district of Japan and tested for AGID antibody, the rate of positive reactors was 57.5%. Positive reactors were found to account for more than 50% of the samples from each prefecture (Table 1). Serum samples were grouped according to the age of the donor cattle. Positive reactors gradually increased in number with age and there was no negative reactor among cattle more than 5 years old (Table 2).

Antibody survey of other domestic animals: An antibody survey was conducted on serum samples of other domestic animals. No antibody-positive samples were detected among horses and swine, but more than 80% of the sheep examined were positive reactors (Table 1).

DISCUSSION

The isolated virus, temporarily named the Chiba strain, was identified as a member of the genus parapoxvirus in the family Poxviridae on the basis of the physicochemical properties of the virus, electron microscopic observation and serological tests in the present study. The genus parapoxvirus is divided into several species according to animals naturally infected and clinical symptoms [2]. According to this classification, the Chiba strain would be a pseudocowpox virus or milker's node virus [2, 9], but all parapoxviruses are immunologically closely related and cannot be distinguished in cross-neutralization tests [17]. It is therefore a matter of controversy whether these viruses should be regarded as variants of one virus which has adapted to different hosts. Recently, distinction was achieved by DNA restriction analysis and hybridization [3, 17]. Previously, parapoxviruses have been isolated from cattle, sheep and the Japanese serow in Japan [1, 7, 11, 12]. The relationships between the Chiba strain and other previously isolated viruses must be investigated by DNA analysis.

There is no report which describes a serological survey of parapoxviruses in Japan, probably because of difficulty in doing the virus neutralization test. The AGID test used

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**Fig. 1.** Virus particles of isolate (Chiba strain) grown in fetal bovine muscle cell cultures. Bar=200 nm.

**Fig. 2.** Demonstration of antigenic identity between isolated virus (1) and previously isolated parapoxviruses (2–5). A precipitation line formed between the control antiserum (S) and isolated virus was connected with the precipitation lines formed by other parapoxviruses (Aomori (2), Iwate (3), Ishikawa-B (4) and Ishikawa-S (5) strains), indicating reaction of identity. No precipitation line was formed between the control antiserum and vaccinia virus (6).

**Table 1.** Survey of antibody to Chiba strain among domestic animals in Japan

<table>
<thead>
<tr>
<th>Animal</th>
<th>Area</th>
<th>Samples tested</th>
<th>Samples positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>Chiba</td>
<td>110</td>
<td>56 (50.9)</td>
</tr>
<tr>
<td></td>
<td>Ibaraki</td>
<td>50</td>
<td>36 (72.0)</td>
</tr>
<tr>
<td></td>
<td>Gunma</td>
<td>59</td>
<td>34 (57.6)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Hokkaido</td>
<td>52</td>
<td>46 (88.5)</td>
</tr>
<tr>
<td>Horse</td>
<td>Ibaraki</td>
<td>50</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Pig</td>
<td>Chiba</td>
<td>162</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>
in this study is a simple and reliable serological test for parapoxvirus. Since the precipitation lines formed by the parapoxvirus strains connected to each other and no precipitation line was formed by FBM cells infected with vaccinia virus, the precipitation line was specific for parapoxvirus but not produced nonspecifically by FBM cells. The present study indicated that there is a high percentage of antibody positive cattle in the Kanto district in Japan and the positive rate increases with age. Though more than 50% of cattle were infected with parapoxviruses, epidemics of the disease among them have rarely been reported. The symptoms of parapoxvirus infection were usually not so severe and many cattle were infected subclinically, but a disease which might be due to parapoxvirus infection has been sporadically reported among cattle in Japan recently. It is not clear why this disease has begun to be observed. There are two possible reasons. One possibility is the change in concern by veterinarians and farmers. After the outbreak of foot-and-mouth disease in Japan, they have become careful in observing their cattle, especially around the mouth and teats. They are therefore anxious about symptoms which might have passed unnoticed before. Another possibility is the increase in the number of infected cattle. When a Japanese serow with papular stomatitis was found in 1979, a seroepidemiological survey was performed on antibody to the isolated parapoxvirus in cattle on several farms in Aomori Prefecture, and the rate of positive reactors was 37% (Takatori et al., Annu. Meet. Jpn. Soc. Vet. Sci. 89th, 1980), but at present, most cattle have antibodies to parapoxvirus. This phenomena is probably due to a change in farming style. Once, a newborn calf was brought up to a cow on the same farm, but now breeding farms and dairy farms are specialized. Young cattle are transferred from a breeding farm to a dairy farm. The increase in the number of cattle transported also seems to work as a factor in the prevalent infection of parapoxvirus. The number of farmers who are self-sufficient in roughage is also decreasing and many have begun to get it from outside. Since parapoxvirus is extremely stable and contaminated pasture remains infectious for many months [10], the roughage might work as a source of infection when carried to a farm.

Viral exchange between cattle and wild animals is also considerable. It is known that the Japanese serow is prevalently infected with parapoxvirus [16]. Affected Japanese serows are often captured and the characteristic virus particles are observed in their lesions [13]. Therefore, it is quite likely that wild animals play an important part in the epidemiology of parapoxvirus infection. A high percentage of antibody positive reactors were also observed among sheep in Hokkaido. As far as we know, no clinical evidence of parapoxvirus infection has been found among sheep in Hokkaido, but the healthy sheep in Hokkaido might be infected subclinically with a certain virus strain in genus parapoxvirus and viral exchange between cattle and sheep would be possible.

Parapoxvirus infection is not serious except for the Japanese serow, which suffers from papular stomatitis with high mortality, but parapoxvirus infection in a milking herd sometimes induce pustules and ulcers on teats of cattle and results in economic losses to farmers. Furthermore, the clinical symptoms look like those induced by serious exotic diseases such as foot-and-mouth disease. Therefore, the prevalence of parapoxvirus among cattle and other domestic animals must be investigated. A rapid and reliable method for diagnosis of this disease is also required.

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