NOTE *Pathology*

Mammary Lesions Associated with Bovine Herpesvirus Type 4 in a Cow with Clinical Mastitis

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ABSTRACT. Intranuclear eosinophilic inclusion bodies were seen in the lactiferous duct and sinus epithelium of mammary tissues collected from a cow with clinical mastitis. Transmission electron microscopy revealed herpesvirus particles in these cells. Immunolabeling against anti bovine herpesvirus type 4 (BHV-4) rabbit serum was detected in nuclei that had intranuclear inclusion bodies. In addition, BHV-4 was isolated from the mammary tissue. The viral DNA was detected by nested PCR from the same tissue. This is the first report to describe mammary lesions in association with BHV-4.
KEY WORDS: BHV-4, mastitis, pathology.

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Clinical mastitis is the disease with the largest economic impact on the dairy industry. Despite intensive bacteriological research, 20 to 35% of clinical cases of bovine mastitis have an unknown aetiology [2, 8]. Although virus infection was suspected in such cases, the role of viruses in bovine mastitis has been generally ignored until Wellenberg *et al.* [10] isolated bovine herpesvirus type 4 (BHV-4) in milk from cows with clinical mastitis in 2000. These samples did not, however, demonstrate an association between BHV-4 and mammary lesions in either natural cases [10] or experimental infections with BHV-4 isolate [9]. Our study results do demonstrate the possible role of BHV-4 in bovine clinical mastitis.

In December 2001, a 6-year-old Holstein cow developed a fever (41.3°C) a few days after parturition, and stopped milking with mammary consolidation. Despite treatment with antibiotics and ointment for mastitis, the cow died 15 days after parturition. Postmortem examination was carried out 3 days after the death and was concentrated on the udder. No lesion was seen in the skin of the udder or the teats, though the lower surface of the udder had turned to dark green. Palpation of the udder revealed moderate consolidation. On the cut surface, the mammary tissue was partially autolysed and half-liquefied. Dilation of lactiferous sinuses and ducts can be seen with mild autolysis, and the sinuses and ducts were filled with milk-yellow exudate. The mammary tissues including lactiferous ducts were collected and fixed in buffered 20% formalin, dehydrated in alcohol, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The other organs were not collected due to severe autolysis.

Histologically, autolytic changes were not too severe to recognize microscopic lesions in some areas of the mammary tissues, though the autopsy was carried out 3 days after the cow's death. In such areas, most of the lactiferous ducts and sinuses were filled with debris containing degenerated epithelium, neutrophils, and clumps of bacilli (Fig. 1). The epithelial cells were degenerating and desquamating. Some of them had large swollen nuclei with eosinophilic inclusion bodies surrounded by a clear halo (Fig. 2). There were focal squamous metaplasia in the sinus and ductal epithelium. Intranuclear inclusion bodies were sometimes seen in these metaplastic cells. Tunica propria, and interlobular connective tissue were moderately dilated with congestion, edema, and infiltration of mononuclear cells and neutrophils. There was mild to moderate neutrophilic infiltration in mammary acini. No inclusion body was seen in the acinar cells.

Streptavidin biotin (SAB) technique was performed as described [7] on paraffin sections. The reagent, except for primary antibodies, was supplied in a commercial kit (Histofine SAB, Nichirei, Tokyo, Japan). We used mouse anti BHV-1 monoclonal antibody (diluted at 1:2048, VMRD, U.S.A.) and rabbit anti BHV-4 serum (diluted at 1:128, NIAH, Ibaraki, Japan) as the primary antibody. As a result, immunolabeling against BHV-4 antiserum was seen in nuclei in which intranuclear inclusion bodies existed (Fig. 3). No positive reaction was observed with anti BHV-1 antibody.

For transmission electron microscopy, formalin-fixed paraffin-embedded mammary tissue samples were deparaffinized in xylene, rehydrated with a series of alcohol, and then washed in 0.1 M phosphate buffer (PB, pH 7.4). The sample were refixed in 2.5% glutaraldehyde in PB, post-fixed in 1% osmium tetroxide, and embedded in epoxy resin. Ultra-thin sections were double-stained with uranyl acetate and lead and observed under a transmission electron microscope (Hitach-7500, Hitachi Co., Ltd., Ibaraki, Japan). The observation revealed virions in epithelial cells having

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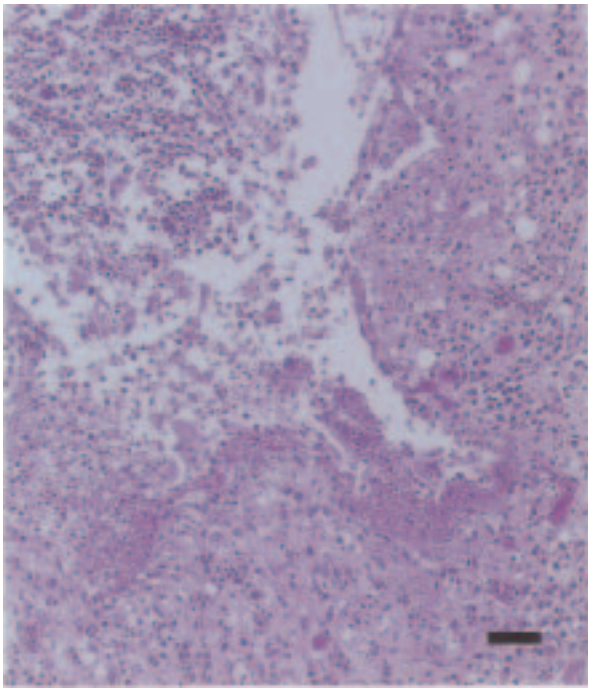

Fig. 1. Mammary tissue of a 6-year-old cow. A lactiferous duct was filled with debris containing degenerated epithelium, neutrophils, and clumps of bacilli. Hematoxylin and eosin (HE) stain. Bar=50 μm.

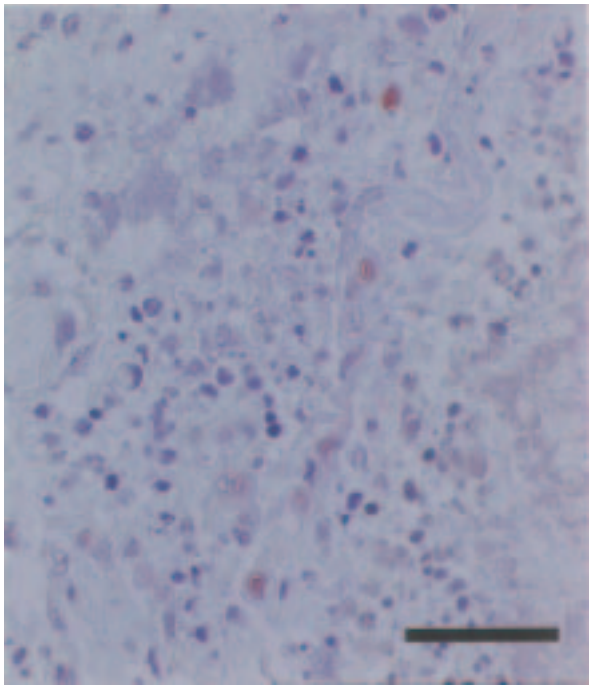

Fig. 3. Immunolabeling against BHV-4 antiserum was seen in nuclei in which intranuclear inclusion bodies existed. Immunostaining. Bar=50 μm.

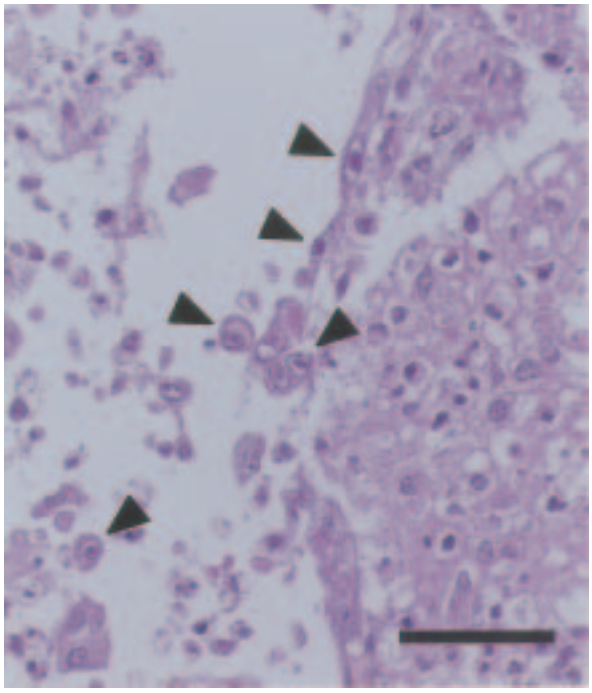

Fig. 2. The epithelial cells of the lactiferous duct were degenerating and desquamating. Some of them had large swollen nuclei with eosinophilic inclusion bodies surrounded by a clear halo (arrowheads). HE stain. Bar=50 μm.

intranuclear inclusion bodies. Immature virions approximately 100 nm in diameter, each of which consisted of a core and capsid, were seen in the nuclei. Mature virions 150-160 nm in diameter, which consisted of nucleocapsids and envelopes, were seen in the cytoplasm (Fig. 4). The morphology of the virus agreed with that of herpesvirus.

Since histopathological examination and electron microscopic observation indicated that the mastitis was related to BHV-4 infection, virus isolation was performed using the mammary tissue. The frozen tissue was thawed and cut into pieces, suspended in 9 volumes of Eagle's minimal essential medium (Eagle MEM), homogenized and centrifuged at 1,000 g for 20 min. The supernatant was passed through the 450 nm filter and inoculated onto Madin-Darby bovine kidney (MDBK) cells cultivated in Eagle MEM containing 5% fetal calf serum, 0.3% triptose phosphate broth , 100 μg/m*l* of streptomycine and 100 μ/m*l* of penicillin. The inoculated cells showed signs of cytopathic effect (CPE) at 7 days after inoculation. The CPE was more obvious and appeared within 72 hr after passage three in MDBK cells. DNA was extracted from the infected cells using a commercially available test kit (SepaGene, Sankoujunyaku Co., Ltd., Tokyo, Japan) and isolation of BHV-4 was ascertained by PCR and nested PCR [5]. The sequences of the first PCR primers were 5'-GTTGGGCGTCCTGTATGGTAGC-3' and 5'-ATGTATGCCCAAAACTTATAATATGACCAG-3', and a 567-bp product was predicted. The sequences of the second PCR primers were 5'-TTGATAGTGCGTTGTTGG-

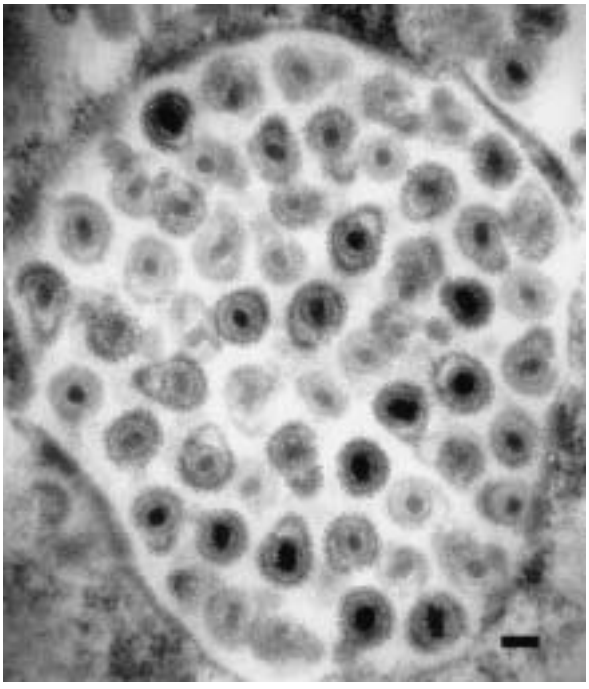

Fig. 4. Electron micrograph of a desquamated epithelial cell of a lactiferous duct. Mature herpesvirus were seen in the cytoplasm. Bar=100 nm.

GATGTGG-3' and 5'-CACTGCCCGGTGGGAAATAGC A-3', and this round of PCR amplified a 260-bp product. The specificity of the PCR products was confirmed by restriction fragment length polymorphism (RFLP) [1, 12]. Nested PCR was also performed using DNA sample extracted from paraffin sections of the mammary tissue. The extraction of DNA was performed using a commercial kit (PUREGENE DNA Isolation Kit, Genetica Systems, U.S.A.). The expected size of DNA was not detected by first PCR but produced by the nested PCR and the specificity of the DNA product was also confirmed by RFLP.

For bacteriological examination, mammary tissues were emulsified and prepared in a ten-fold dilution with series of phosphate-buffered saline solution (pH 7.4). One to 10 m*l* of each dilution was spread onto 5% ovine blood agar. The plates were incubated aerobically or anaerobically for 48 hr at 37°C. No significant bacteria, however, was isolated.

Although a few investigators described isolation of BHV-4 [4] or detection of the viral DNA [3, 11] from milk of cows with clinical mastitis, no one has reported either histopathological changes of mammary tissue associated with BHV-4 or in situ detection of BHV-4. In the present study, we detected intranuclear inclusion bodies in the mammary tissues of a cow with clinical mastitis. Immunohistochemistry could be successfully used to detect BHV-4 antigen, and electron microscopy revealed herpesvirus particles in the cells with inclusions. Infection of BHV-4 was also demonstrated by virus isolation and nested PCR technique. This is the first report of an association of BHV-4 with mammary

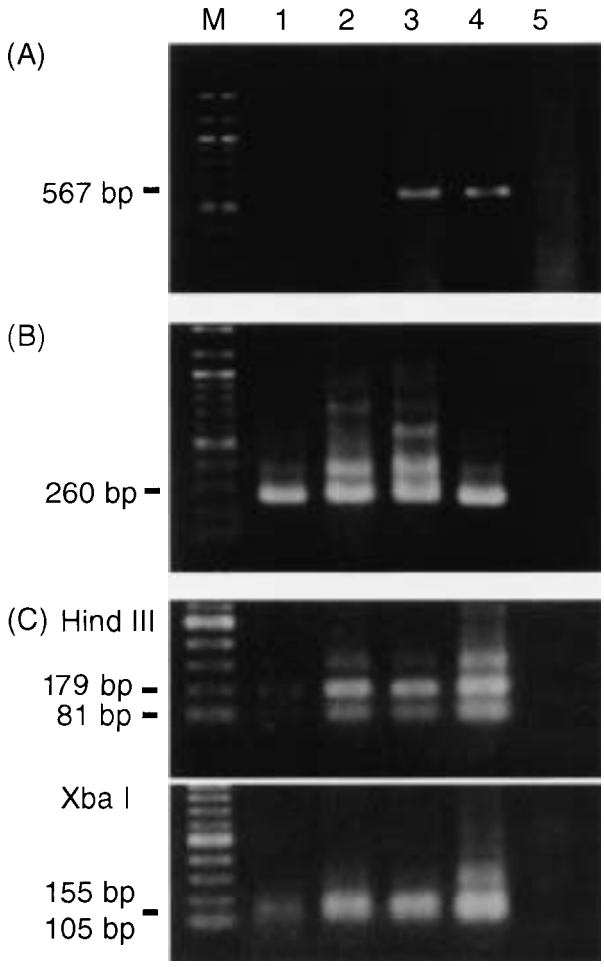

Fig. 5. Detection of BHV-4 from the mammary lesions. (A) First PCR. (B) Nested PCR. (C) PCR-RFLP analysis. A 260-bp products were respectively cut by *Hind* III (81 and 179 bp) and *Xba* I (105 and 155 bp). Lane: M, 100 bp ladder; 1, paraffin-embedded mammary sample; 2, mammary tissue; 3, MDBK cells infected with isolated virus; 4, MDBK cells infected with strain B11–41; 5, MDBK cells.

lesions in a cow with mastitis.

It could not be definitively determined whether BHV-4 was a primary infection or was secondary to bacterial mastitis. The fact that inclusion bodies appear for only a transient period 2–3 days after experimental respiratory infection of BHV-1 [4] may support the latter scenario. The possibility remains, however, that BHV-4 is a primary and persistent infection, as occurs in field cases of BHV-1 infection, in which inclusion bodies occasionally persist long enough to be found in bronchial or alveolar epithelium [4]. The primary BHV-4 infection may facilitate secondary bacterial infection.

BHV-4 was associated with degeneration and desquamation of epithelial cells. These lesions were principally similar to those seen in endometrium of cows naturally infected

with BHV-4 [5]. It was not clear whether BHV-4 infection caused squamous metaplasia in the sinus and ductal epithelium. Although no bacteria were isolated, probably due to the treatment with antibiotics, suppurative inflammation in the present case was most likely caused by bacterial infection.

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