Studies on the infectious diseases of cattle in Yamaguchi

山口県における牛の感染症に関する研究

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September 2018

1. GENERAL INTRODUCTION

1.1. Infectious diseases in cattle

Infectious diseases in cattle are caused by various pathogens such as viruses, bacteria, fungi, protozoa and parasites. Although vaccine and antibiotics are administrated to cattle for the prevention and treatment of infectious diseases, these have not yet been well controlled. The infectious diseases cause weight loss, decrease of lactation and death, resulting in big economic loss in farmers.

In Japan, Act on “Domestic Animal Infectious Diseases Control (Act No. 166 of 1951)” was established for prevention of diseases in livestock animals. In this law, 28 domestic animal infectious diseases and 71 notifiable infectious diseases are prescribed.

Since 1950, livestock hygiene service centers were established in each prefectures based on “Livestock Hygiene Service Centers Act (Act No. 12 of 1950)”, and have contributed for prevention and diagnosis of infectious diseases in livestock. In Yamaguchi Prefecture, four livestock Hygiene Service Centers (Toubu, Chubu, Seibu and Hokubu) were established, and have played important roles in diagnosis and prevention of infectious diseases in livestock in Yamaguchi.

2. CHAPTER 1

Infectious diseases in cattle in Yamaguchi Prefecture from 2006 to 2016

2.1. ABSTRUCT

The infectious diseases and pathogens detected in cattle in Yamaguchi Prefecture from 2006 to 2016 were summarized in order to understand the current status and to prevent the infectious diseases. From 2006 to 2016, among cattle, only a few cases were reported for infectious diseases designated as domestic animal infectious diseases and notifiable infectious diseases. However, the five infectious diseases, Akabane disease, bovine viral diarrhea-mucosal disease, infectious bovine rhinotracheitis, bovine leukemia and tetanus, were reported and their annual incidence was higher than those in other prefectures. Also, large numbers of opportunistic infectious diseases were reported among cattle. The ratio of infectious diseases was the highest in meat calves (51.7%), followed by dairy cattle (23.2%), fattening cattle (10.8%), milk calves (7.6%) and breeding cattle (6.7%). The ratio of microorganisms casused infectious diseases in Yamaguchi cattle were 72.1%, 21.9%, 2.8%, 1.9%, and 0.3% in bacteria, viruses, protozoa, fungi and parasites, respectively.

2.2. BACKGROUND

As of February 1st, 2016, a total of 19,593 (16,476 beef cattle and 3,117 dairy cattle) are bred in Yamaguchi Prefecture (website of Yamaguchi Prefectural Government, http://www.pref.yamaguchi.lg.jp/cms/a17600/chikuchou/chikuchou.html).

As domestic animal infectious diseases, sixteen cases of Johne's disease were observed by periodic inspection based on article 5 of Act on Domestic Animal Infectious Diseases Control in 2006 and 2007, but has not been reported since 2007. Johne's diseases occurred nationwide and 7,311 cases (except Yamaguchi Prefecture) were confirmed from 2006 to 2016. For other domestic animal infectious diseases, 15 transmissible spongiform encephalopathy, 9 foot-and-mouth disease, 5 brucellosis, 5 tuberculosis, 2 epidemic encephalitis and 2 anaplasmosis occurred in Japan (website of Ministry of Agriculture, Forestry and Fisheries of Japan, http://www.maff.go.jp/j/syouan/douei/kansi_densen/kansi_densen.html), while no report of any of those infectious diseases in Yamaguchi prefecture from 2006 to 2016.

As notifiable infectious diseases, 220 cases of were reported in Yamaguchi Prefecture; 176 bovine leukosis, 17 bovine viral diarrhea-mucosal disease, 14 Akabane disease, 11 infectious bovine rhinotracheitis and 2 tetanus.

To understand infectious diseases in cattle in Yamaguchi Prefecture, the data of diagnosis of infectious diseases performed in Chubu Livestock Hygiene Service Center were summarized in this chapter.

2.3. MATERIALS AND METHODS

The ratio of cases of notifiable infectious diseases in Yamaguchi Prefecture was calculated and compared with the results in the other prefectures. Since diagnostic method of Johne's disease varied in each prefecture, the data is not analyzed. 806 cases were examined in Yamaguchi Chubu Livestock Hygiene Service Center and pathogens were identified in 462 cases. In these 462 cases, clinical signs, date of onset, species of pathogens were analyzed.

2.4. RESULTS

2.4.1. Notifiable infectious diseases in Yamaguchi Prefecture from 2006 to 2016

Table 2-1 shows five notifiable infectious diseases that occurred sporadically in Yamaguchi Prefecture, Akabane disease, bovine viral diarrhea-mucosal disease, infectious bovine rhinotracheitis, bovine leukemia and tetanus. Bovine leukemia have been reported every year from 2006 to 2016, and the similar trend was observed nationwide. For these diseases, the annual incidence in Yamaguchi tended to be higher than those in other prefectures.

2.4.2. Analysis of the cases of infectious diseases by month and year

The proportion of infectious diseases cases was the highest in meat calves (51.7%), followed by dairy cattle (23.2%), fattening cattle (10.8%), milk calves (7.6%) and breeding cattle (6.7%) (Figure 2-1).

The numbers of infectious diseases were ranged from 28 up to 65 cases per year from 2006 to 2016, increased to over 50 cases between 2011 and 2013, and then decreased to less than 30 cases between 2014 and 2016 (Figure 2-2)**.** The numbers of infectious diseases were the lowest in June and were the highest in December, followed by October, August, and April (Figure 2-3).

The most observed microorganisms causing infectious diseases in Yamaguchi prefecture was bacterial from 2006 to 2016, followed by virus and protozoa as described below. Also, co-infections were also observed in many cases (Figure 2-4). By month, bacterial infections were observed in August the most and viral infections were in October and December (Figure 2-5).

2.4.3. Analysis of the cases of infectious diseases by clinical sign

The major clinical sings (77.1%) observed in diseased cattle were these four signs; 164 cases with respiratory sign (27.6%), 118 cases of death (19.8%), 102 cases with diarrhea (17.1%), and 75 cases with mastitis (12.6%) (Table 2-2).

In meat calves, 121 cases with respiratory sign (35.3%), 92 cases of death (26.8%), and 63 cases with diarrhea (18.4%) were observed. These three clinical signs were major in meat calves (80.5%). In milk calves, 13 cases with diarrhea (27.1%), 11 cases with respiratory sign (22.9%), 9

cases of death (18.8%) and 5 cases with astasia (10.4%) were observed. In breeding cattle, 9 cases of stillbirth (25.7%), 7 cases of abortion (20.0%) and 6 cases with diarrhea (17.1%) were observed. In dairy cattle, 75 cases with mastitis (67.6%), 13 cases of diarrhea (11.7%) and 6 cases with respiratory sign (5.4%) were observed. In fattening cattle, 24 cases with respiratory sign (41.4%), 16 cases of death (27.6%) and 7 cases with diarrhea (12.1%) were observed. Respiratory signs were the most observed in meat calves (35.3%) and fattening cattle (41.4%). Diarrhea was observed in more than 10% of cattle (Table 2-3).

2.4.4. Analysis of the cases of infectious diseases by pathogens

Five hundred fifty-nine cases of bacterial (72.1%), 170 cases of viral (21.9%), 22 cases of protozoan (2.8%), 15 cases of fungal (1.9%) and 2 cases of parasitic (0.3%) infection were observed in Yamaguchi prefecture from 2006 to 2016. The ratio of infection with bacteria and/or virus was 94.1% (Figure 2-6). In diagnosis by only histopathological examinations, there were 7 cases (0.9%) with suspected contribution of pathogens.

For bacterial infections, 169 cases of *Mycoplasma* (30.2%), 58 cases of *Staphylococcus* (10.4%), 50 cases of *Pasteurella* (8.9%), 35 cases of *Escherichia coli* (6.3%), 33 cases of *Streptococcus* (5.9%) and *Clostridium* was in 31 cases (5.5%) were observed (Table 2-4). Among 169 cases of *Mycoplasma* infection, *M. dispar*, *M. bovis*, *M. bovirhinis* and *M. bovigenitalium* were observed in 75 (44.4%), 49 (29.0%), 39 (23.1%) and 2 (1.2%) cases, respectively. Among 58 cases of *Staphylococcus* infection, *S. aureus*, *S. chromogenes*, *S. simulans* and *S. hyicus* were observed in 15 (25.9%), 9 (15.5%), 9 (15.5%) and 5 (8.6%) cases, respectively. Among 50 cases of *Pasteurella* infection, *P. multocida* was observed in 45 cases (90.0%). Among 33 cases of *Streptococcus* infection, *S. uberis*, *S. bovis* and *S. dysagalactiae* were observed in 16 (48.5%), 11 (33.3%) and 3 (9.1%) cases, respectively. Among 31 cases of *Clostridium* infection, *C. perfringens* was observed in 22 cases (71.0%). For viral infection, 47 cases of bovine coronaviruses (BCV) (27.6%), 23 cases of bovine respiratory syncytial virus (BRSV) (13.5%), 18 cases of Akabane virus (AKAV) (10.6%), 13 cases of bovine viral diarrhea virus (BVDV) (7.6%) and 13 cases of rotavirus A (GAR) (7.6%) were observed (Table 2-4). For protozoan infection, 18 cases of *Eimeria* (81.8%), 3 cases of

Cryptosporidium parvum (13.6%), and 1 case of *Neospora caninum* (4.5%) were observed (Table 2-4). For fungal infection, 6 cases of *Candida* (40.0%), one case of *Aspergillus Niger*, *Absidia* and *Penicillium* (20.0%) were observed. Also, infection of fungi was suspected in 6 cases (40.0%) by histopathological examinations (Table 2-4). For parasitic infection, one case of *Fasciola* and *Strongyloides papillosus* were observed (Table 2-4).

2.4.5. Analysis of the cases of infectious diseases by pathogens and use of cattle

Among meat calves, for bacterial infection, 141 cases of *Mycoplasma*, 39 cases of *Pasteurella*, 23 cases of *E. coli*, 15 cases of *Clostridium* and *Ureaplasma diversum* were observed. For viral infection, 25 cases of BCV, 11 cases of BRSV, 10 cases of BEV and 10 cases of AKAV were observed. For fungal infection, *Candida* (5 cases) were observed most frequently. For protozoan infection, 15 cases of *Eimeria* and 3 cases of *Cryptosporidium parvum* were observed. For parasitic infection, only one case of *Strongyloides papillosus* was observed (Table 2-5). Among milk calves, for bacterial infection, 11 cases of *Mycoplasma* and 7 cases of *Clostridium* were observed. For viral infection, 4 cases of AKAV, 3 cases of BCV and 3 cases of BVDV were observed. For protozoan infection, 2 cases of *Eimeria* were observed. No Fungal or parasitic infection was observed in milk calves (Table 2-5). Among breeding cattle, for bacterial infection, 6 cases of *Mycoplasma* and 3 cases of *E. coli* were observed. For viral infection, 3 cases of BLV, 2 cases of BVDV, 2 cases of BRSV and 2 cases of BCV were observed. For protozoan infection, one case of *Neospora caninum* was observed. For parasitic infection, one case of *Fasciola* was observed. No Fungal infection was reported in breeding cattle (Table 2-5). Among dairy cattle, for bacterial infection, 58 cases of *Staphylococcus*, 31 cases of *Streptococcus* and 11 cases of *Corynebacterium* were observed. For viral infection, 10 case of BCV and 3 cases of BLV were observed. For fungal infection, one case of *Candida terebra* and *fungus* were observed. No protozoan or parasitic infection were observed in dairy cattle (Table 2-5). Among fattening cattle, for bacterial infection, 9 cases of *Mycoplasma* and 7 cases of *Clostridium* were observed. For viral infection, 8 cases of BRSV, 7 cases of BCV and 6 cases of BPIV-3 were observed. For protozoan infection, only one case of *Eimeria* was observed. No fungal or parasitic infection were observed among fattening cattle (Table

2-5).

2.4.6. Analysis of the cases of infectious diseases by pathogens and clinical sign

Pathogens detected in four clinical signs (respiratory sign, deaths, diarrhea and mastitis) and the numbers of cases were shown in Table 2-6.

As cause of respiratory sign, for bacterial infection, 160 cases of *Mycoplasma*, 48 cases of *Pasteurella*, and 19 cases of *Mannheimia haemolytica* were observed (Table 2-6). For viral infection, 24 cases of BCV, 23 cases of BRSV and 9 cases of BPIV-3 were observed. For fungal infection, one case of *Absidia, Aspergillus niger* and *Penicillium* were observed. No protozoan or parasitic infection were observed among cattle showing respiratory sign. As causes of death, for bacterial infection, 26 cases of *Mycoplasma*, 25 cases of *Clostridium* and 15 cases of *E. coli* were observed (Table 2-6). For viral infection, 6 cases of BCV, 6 cases of GAR, and 4 cases of BVDV were observed. For fungal infection, 2 cases of *Candida*, 1 case of *Absidia*, 1 case of *Aspergillus niger*, 1 case of *Penicillium* and 5 cases of *fungi* were observed. For protozoan infection, 10 cases of *Eimeria* and 2 cases of *Cryptosporidium parvum* were observed. For parasitic infection, only one case of *Strongyloides papillosus* was observed. As causes of diarrhea, for bacterial infection, 12 cases of *E. coli*, 9 cases of *C. perfringens* and 2 cases of *Chlamydia pecorum* were observed (Table 2-6). For viral infection, 27 cases of BCV, 13 cases of GAR, 12 cases of BEV and 9 cases of BVDV were observed. For fungal infection, 2 cases of *Candida* and 3 cases of *fungi* were detected. For protozoan infection, 17 cases of *Eimeria* and 2 cases of *Cryptosporidium parvum* were observed. For parasitic infection, 1 case of *Fasciola* was observed. As causes of mastitis, for bacterial infection, 58 cases of *Staphylococcus*, 29 cases of *Streptococcus*, and 10 cases of *Corynebacterium* were observed (Table 2-6). For fungal infection, *Candida terebra* and *fungi* were detected one by one.

2.5. DISCUSSION

Among cattle in Yamaguchi prefecture from 2006 to 2016, the number of infectious diseases as domestic animal infectious diseases and notifiable infectious diseases were very small. On the other hand, it has been found that there were many opportunistic infectious diseases that caused significant economic loss to farmers by diarrhea, respiratory sign and mastitis.

Based on the questionnaire survey for clinical veterinarians, mastitis, diarrhea and pneumonia were reported to be the three major clinical signs by infectious pathogens in cattle in Japan (Kiku, 2013). The similar results were observed in cattle in Yamaguchi Prefecture.

Mycoplasma, BCV and BRSV were detected in a large number of cattle, especially calves and fattening cattle with respiratory sign and were major pathogens causing respiratory sign in cattle in Yamaguchi Prefecture. Since *Mycoplasma* is the most frequently detected from death cases, the countermeasures against them should be established immediately.

E. coli, *Clostridium*, BCV, BVDV and GAR were detected from a large number of cattle with diarrhea, suggesting these were major causes of diarrhea in cattle in Yamaguchi prefecture. *E. coli* was detected not only in diarrhea, but also in the other cases such as respiratory sign, neurological sign, mastitis and abortion (Table 2-4). *Clostridium* was mainly detected in sudden death of cattle (Table 2-4, 2-6). BCV was detected not only from diarrhea cases, but also from respiratory signs cases. BVDV was detected from various specimens other than diarrhea cases, such as respiratory sign, diarrhea, and premature birth. Therefore, vaccination for these pathogens may be necessary for cattle in Yamaguchi Prefecture.

Mastitis occurred frequently in dairy cattle with bacterial infection (Table 2-3, 2-6), and *Staphylococcus* and *Streptococcus* were detected the most frequently from specimens of mastitis cases, suggesting that these bacteria are major causes of mastitis in Yamaguchi prefecture. In a few cases, mastitis with suspected contribution of *Mycoplasma* were observed. In general, bovine mastitis caused by mycoplasmas is a major problem for milk production in dairy herds and mycoplasma infection causes severe damage to the udder of cattle and is difficult to be treated by chemotherapy (Nicholas *et al*., 2016). Therefore, even though less cases have been observed in Yamaguchi prefecture, we still need to pay attention also to the mastitis caused by *Mycoplasma*

infection.

Although abortion and stillbirth were observed sporadically, it caused significant economic loss to farmers. Among them, *U. diversum* were detected from lungs and arthritis of several abnormal calves. *U. diversum* is mainly detected from respiratory lesion and rarely detected from abnormal calves. Further studies will be required to clarify whether *U. diversum* causes any diseases in cattle. In several cases, fungi were detected from dead calves, suggesting that calves may be immunosuppressed.

Since the majority of bovine leukemia were observed in slaughterhouse, it is considered that the real number of BLV infection should be more than the number of cases reported. In 2015, Ministry of Agriculture, Forestry and Fisheries has published sanitary measures guidelines on bovine leukemia, and promoted prevention of infection and spreading and the eradication. Since there is no vaccine against this disease, it seems that detection of BLV and isolation or slaughter of infected cattle are the only methods for prevention of spreading BLV. Also, although vaccine is very effective against infectious diseases, cattle should be healthy because vaccination is effective when cattle is healthy. It is important to eliminate various stress factors suppressing immune function of cattle.

Although the cases was not so many, AKAV, *C. pecorum* and PCPV were important pathogens in cattle. In Yamaguchi prefecture, many cases of abortion and stillbirth in cattle by AKAV infection had been reported in the past. In 2007, AKAV was the first isolated in Yamaguchi Prefecture from central nervous system of calf that were infected with AKAV after birth and showed neurological signs (Otani *et al*., 2008). In 2011, multiple cattle developed neurological signs and AKAV was again isolated from their central nervous system (Otani *et al*., 2013).

In 2010, *C. pecorum* was first isolated in Yamaguchi Prefecture from a calf with diarrhea as described below (Ohtani *et al*., 2015) (Table 3-1). Although, *C. pecorum* is one of the pathogens causing sporadic encephalomyelitis, polyarthritis, pneumonia, enterocolitis and keratoconjunctivitis of cattle worldwide (Reinhold *et al*., 2011), pathogenicity of *C. pecorum* remains still unclear in Japan, since there are few reports on *C. pecorum* isolated from cattle in Japan (Fukushi *et al*., 1989; Ohtani *et al*., 2015).

In 2016, a cattle in a farm in Yamaguchi Prefecture showed white vesicles and hyperemia

in mucosa under tongue surface, and pseudocowpox virus (PCPV) was first isolated from the swab of oral lesion in Japan (Ohtani *et al*., 2017). Since clinical signs of cattle infected with PCPV are similar to those of foot-and-mouth disease (FMD), differential diagnosis of PCPV from FMD is required.

In this thesis, establishment of diagnosis method of AKAV, epidemiological study on *C. pecorum* and characterization of PCPV were performed.

2.6. LEGEND FOR FIGURES

Figure 2-1. Analysis of the 462 cases of infectious diseases by use of cattle

Figure 2-2. Analysis of the 462 cases of infectious diseases by year and use of cattle

Figure 2-3. Analysis of the 462 cases of infectious diseases by month and use of cattle

Figure 2-4. Analysis of the 462 cases of infectious diseases by year and pathogens

Figure 2-5. Analysis of the 462 cases of infectious diseases by month and pathogens

Figure 2-6. Analysis of the 462 cases of infectious diseases by pathogens

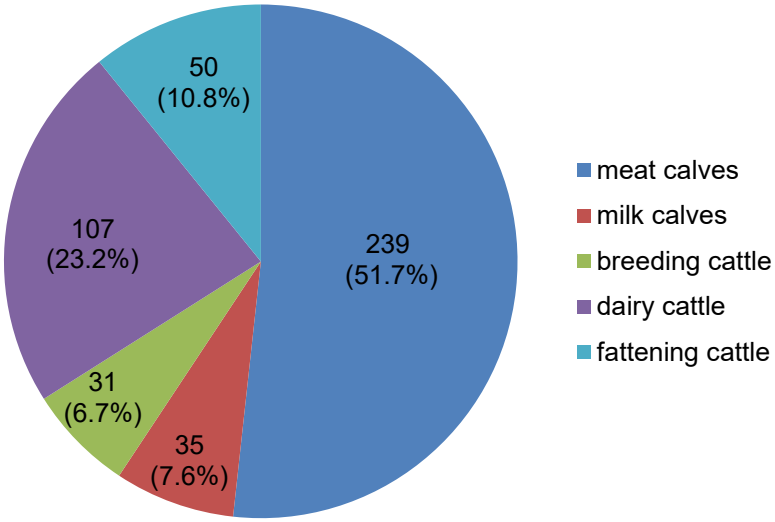

Figure 2-1. Analysis of the 462 cases of infectious diseases by use of cattle

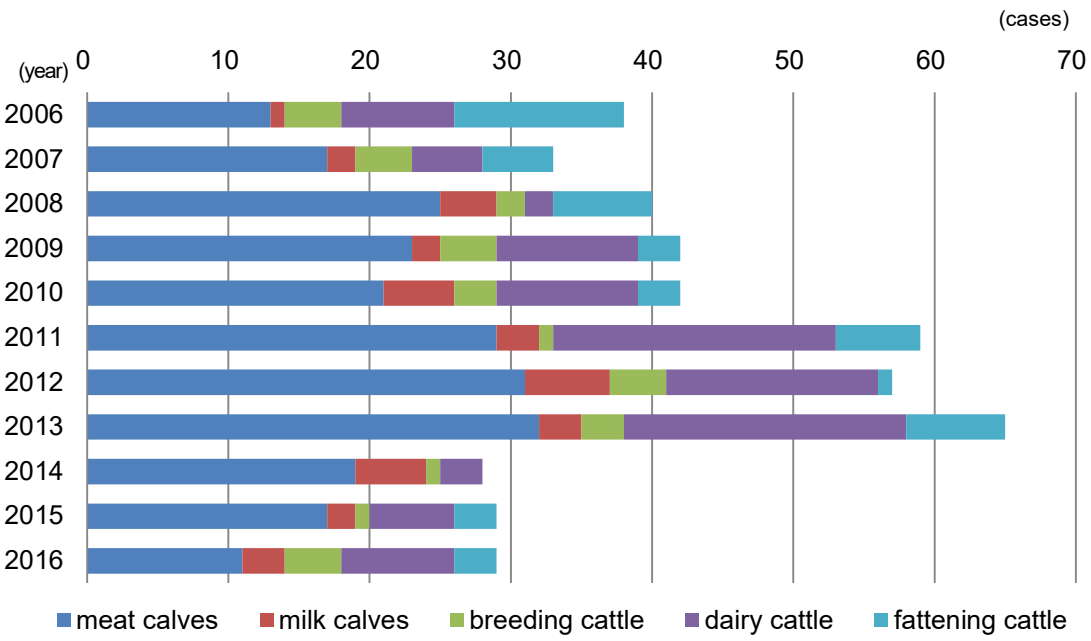

Figure 2-2. Analysis of the 462 cases of infectious diseases by year and use of cattle

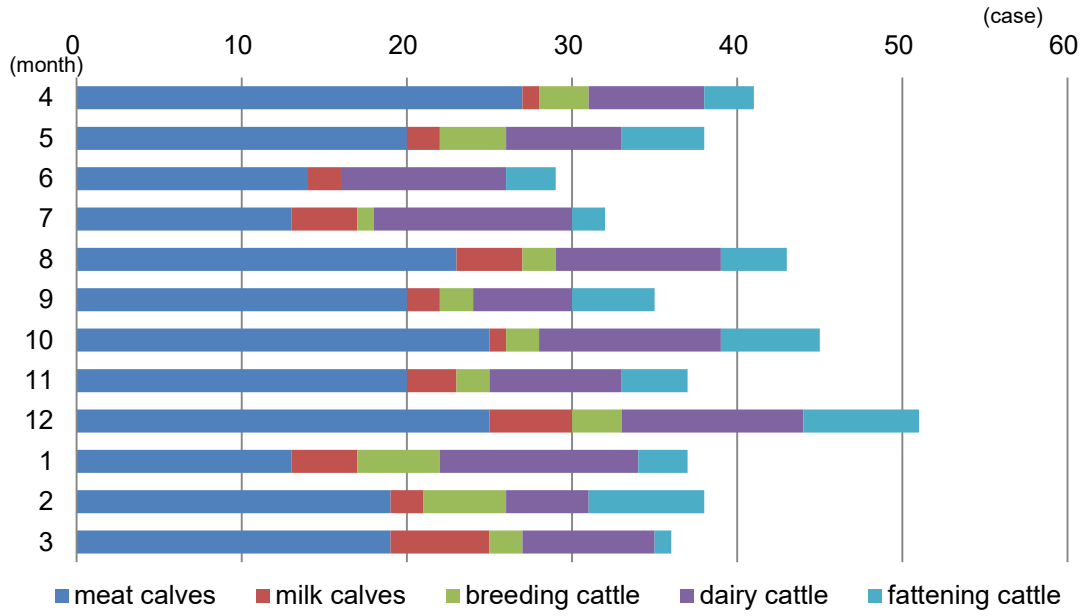

Figure 2-3. Analysis of the 462 cases of infectious diseases by month and use of cattle

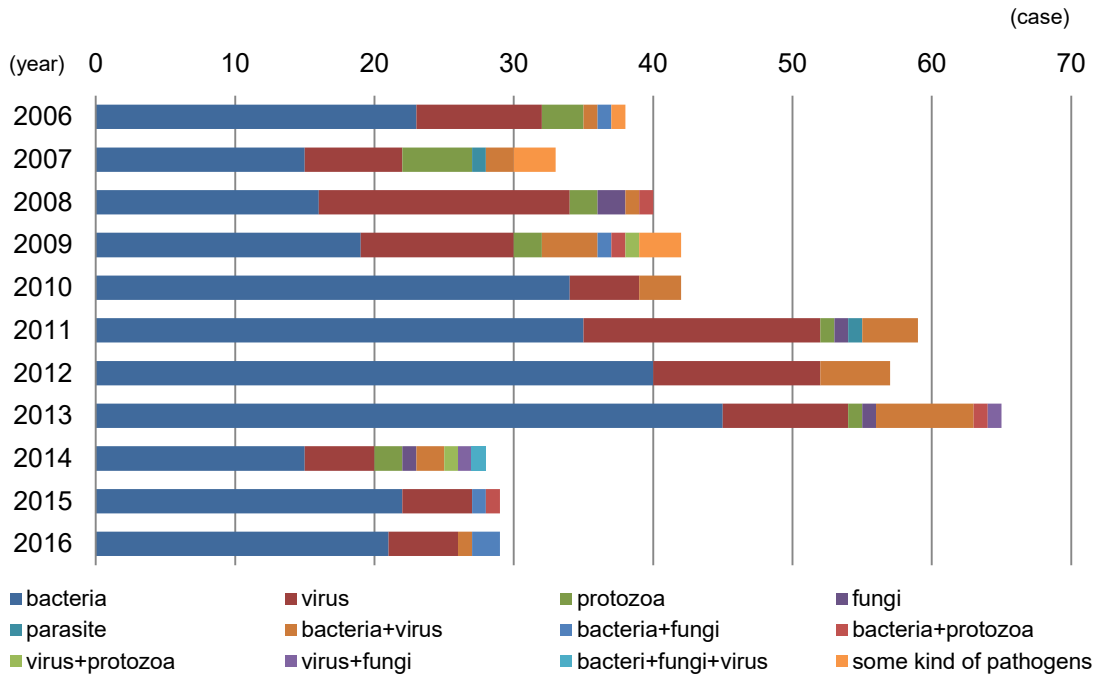

Figure 2-4. Analysis of the 462 cases of infectious diseases by year and pathogens

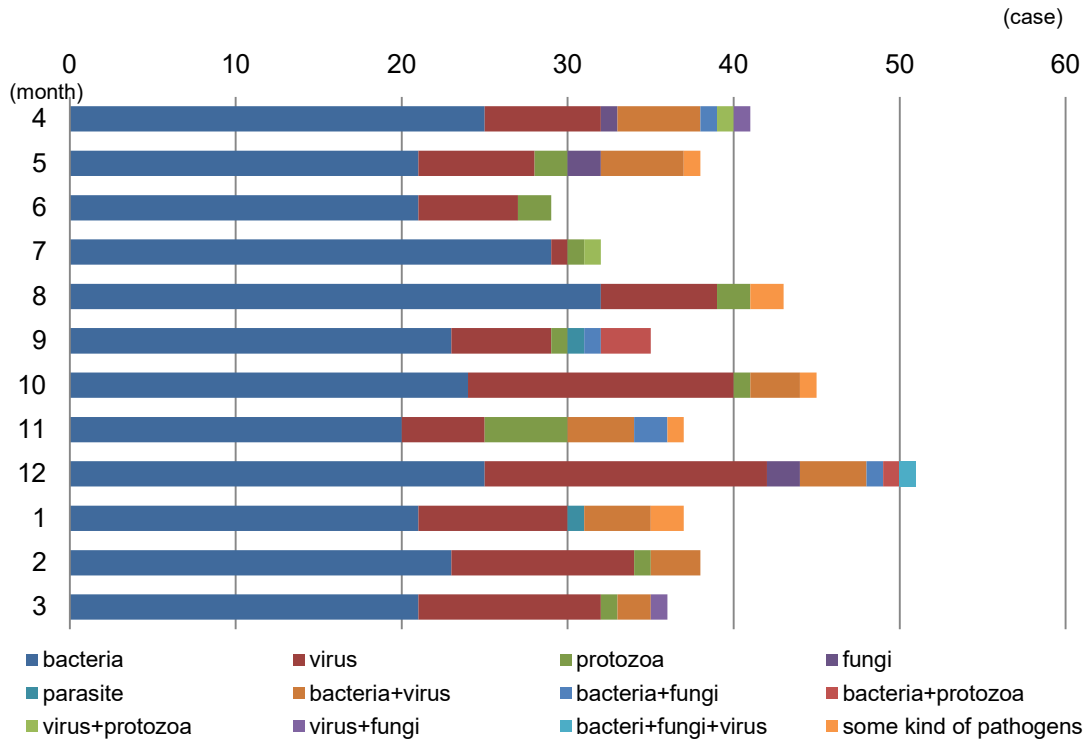

Figure 2-5. Analysis of the 462 cases of infectious diseases by month and pathogens

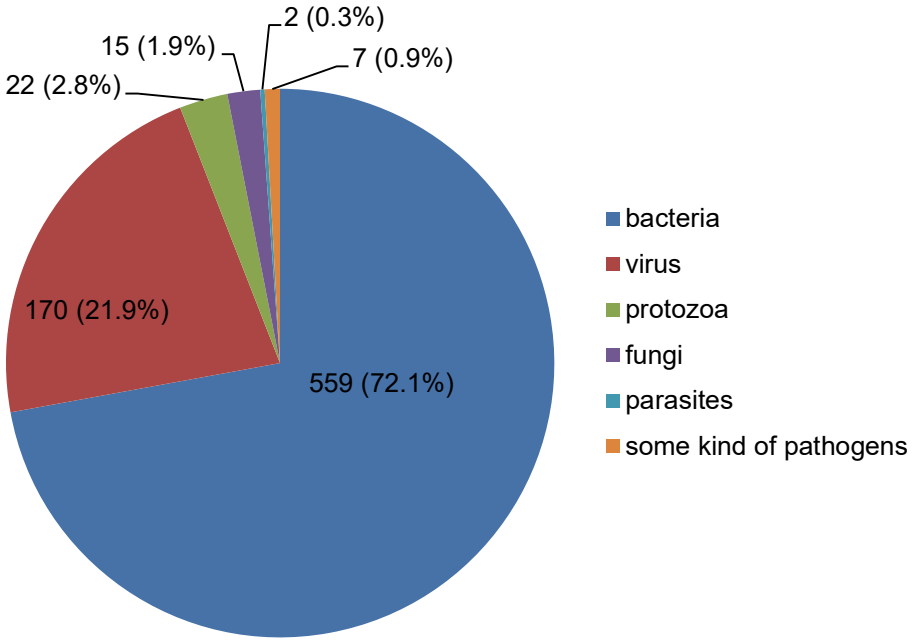

Figure 2-6. Analysis of the 462 cases of infectious diseases by pathogens

Table 2-1 Notifiable infectious diseases occurred in Yamaguchi Prefecture from 2006 to 2016.

Year	Numbers of cattle	Akabane disease			Bovine viral diarrhea-mucosal disease			Infectious bovine rhinotracheitis			Bovine leukemia			Tetanus		
		Cases	Ratio* (%)	Ranking** (n)	Cases	Ratio (%)	Ranking (n)	Cases	Ratio (%)	Ranking (n)	Cases	Ratio (%)	Ranking (n)	Cases	Ratio (%)	Ranking (n)
2006	23,080	0	0	-	1	0.0043	13 (24)	6	0.0260	4 (13)	7	0.0303	17 (46)	0	0	-
2007	23,140	1	0.0043	2 (6)	1	0.0043	7 (16)	0	0	-	3	0.0130	29 (43)	0	0	-
2008	23,330	1	0.0043	11 (14)	1	0.0043	7 (19)	0	0	-	11	0.0471	12 (46)	0	0	-
2009	23,130	2	0.0086	13 (22)	0	0	-	0	0	-	15	0.0649	14 (45)	0	0	-
2010	22,360	0	0	-	0	0	-	0	0	-	9	0.0403	23 (46)	0	0	-
2011	21,280	9	0.0423	6 (16)	0	0	-	0	0	-	19	0.0893	14 (44)	0	0	-
2012	19,570	1	0.0051	2 (4)	0	0	-	0	0	-	30	0.1533	10 (45)	0	0	-
2013	19,260	0	0	-	3	0.0156	4 (17)	5	0.0260	4 (11)	20	0.1038	15 (45)	0	0	-
2014	20,490	0	0	-	9	0.0439	1 (21)	0	0	-	16	0.0781	17 (46)	1	0.0049	6 (15)
2015	19,030	0	0	-	0	0	-	0	0	-	19	0.0998	18 (46)	1	0.0053	4 (21)
2016	18,610	0	0	-	2	0.0107	10 (22)	0	0	-	27	0.1451	16 (46)	0	0	-

* Ratio of the number of diseased cases to the number of cattle bred in Yamaguchi.

** Ranking by the ratio of cases to in comparison with those in the other prefecture.

n: The number of prefectures where the disease occurred.

Table 2-2. Analysis of the 462 cases of infectious diseases by clinical signs

Clinical signs	Cases	Ratio (%)
Respiratory sign	164	27.6
Death	118	19.8
Diarrhea	102	17.1
Mastitis	75	12.6
Astasia	35	5.9
Neurological sign	20	3.4
Abortion	12	2.0
Fragility	10	1.7
Stillbirth	10	1.7
Growth insufficiency	6	1.0
Mass	5	0.8
Loss of appetite	5	0.8
Lameness	5	0.8
Pyrexia	5	0.8
Malformation	4	0.7
Otitis media	4	0.7
Weight loss	2	0.3
Typhlosis	2	0.3
Hemafecia	2	0.3
Arthritis	2	0.3
Premature birth	1	0.2
Decrease of lactation yield	1	0.2
Hematuria	1	0.2
White vesicles on the tongue surface	1	0.2
Torticollis	1	0.2
Sole ulcer	1	0.2
Opacity of eyeball	1	0.2

Table 2-3. Analysis of the 462 cases of infectious diseases by clinical signs and the use of cattle

Meat calves

Clinical signs	Cases	Ratio (%)
Respiratory sign	121	35.3
Death	92	26.8
Diarrhea	63	18.4
Astasia	26	7.6
Neurological sign	12	3.5
Fragility	10	2.9
Growth insufficiency	4	1.2
Otitis media	3	0.9
Lameness	3	0.9
Malformation	2	0.6
Blindness	1	0.3
Pyrexia	1	0.3
Opacity of eyeball	1	0.3
Dysarthrosis	1	0.3
Hemafecia	1	0.3
Weight loss	1	0.3
Arthritis	1	0.3

Milk calves

Clinical signs	Cases	Ratio (%)
Diarrhea	13	27.1
Respiratory sign	11	22.9
Death	9	18.8
Astasia	5	10.4
Neurological sign	3	6.3
Malformation	2	4.2
Otitis media	1	2.1
Blindness	1	2.1
Pyrexia	1	2.1
Torticollis	1	2.1
Growth insufficiency	1	2.1

Breeding cattle

Clinical signs	Cases	Ratio (%)
Stillbirth	9	25.7
Abortion	7	20.0
Diarrhea	6	17.1
Loss of appetite	3	8.6
Respiratory sign	2	5.7
Mass	2	5.7
Death	1	2.9
White vesicles on the tongue surface	1	2.9
Pyrexia	1	2.9
Lameness	1	2.9
Weight loss	1	2.9
Neurological sign	1	2.9

Dairy cattle

Clinical signs	Cases	Ratio (%)
Mastitis	75	67.6
Diarrhea	13	11.7
Respiratory sign	6	5.4
Abortion	5	4.5
Neurological sign	3	2.7
Mass	2	1.8
Decrease of lactation yield	1	0.9
Stillbirth	1	0.9
Premature birth	1	0.9
Astasia	1	0.9
Loss of appetite	1	0.9
Growth insufficiency	1	0.9
Sole ulcer	1	0.9

Fattening cattle

Clinical signs	Cases	Ratio (%)
Respiratory sign	24	41.4
Death	16	27.6
Diarrhea	7	12.1
Astasia	3	5.2
Pyrexia	2	3.4
Hematuria	1	1.7
Hemafecia	1	1.7
Lameness	1	1.7
Mass	1	1.7
Neurological sign	1	1.7
Loss of appetite	1	1.7

Table 2-4. Summary of pathogens

Bacteria (1/2)

Pathogens	Cases	Ratio (%)	Clinical signs
Mycoplasma (M. dispar, bovis, bovirhinis, bovigenitalium, alkalescens, arginine, canadense)	169	30.2	respiratory sign, otitis media, torticollis, mastitis, death
Staphylococcus (S. aureus, chromogenes, simulans, hyicus, warneri, xylosus, captis, cohnii, haemolyticus, lugdunensis, coagulase-negative staphylococci)	58	10.4	mastitis
Pasteurella (P. multocida, trehalosi)	50	8.9	respiratory sign, death, sudden death
Escherichia coli	35	6.3	diarrhea, respiratory sign, neurological sign, mastitis, abortion, stillbirth, death, sudden death
Streptococcus (S. uberis, bovis, dysagalactiae, equinus)	33	5.9	mastitis, abortion, astasia
Clostridium (C. perfringens, sordellii, septicum)	31	5.5	diarrhea, hemafecia, respiratory sign, pyrexia, weight loss, abortion, death, sudden death
Ureaplasma diversum	20	3.6	respiratory sign, abortion, stillbirth, arthritis, death
Mannheimia haemolytica	20	3.6	respiratory sign, death
Trueperella pyogenes	16	2.9	respiratory sign, astasia, mastitis, death, sudden death
Corynebacterium (C. jeikeium, bovis, gulcuronolyticum, macginleyi, propinquum, striatum)	12	2.1	mastitis, sole ulcer, astasia
Histophilus somni	9	1.6	respiratory sign, neurological sign, death, sudden death
Enterococcus (E. faecalis, faecium, gallinarum)	7	1.3	mastitis
Pseudomonas (P. aeruginosa, putida)	7	1.3	respiratory sign, astasia, mastitis, death
Fusobacterium necrophorum	7	1.3	respiratory sign, astasia, death, sudden death
Serratia marcescens	6	1.1	mastitis
Aerococcus (A. viridans, urinae)	5	0.9	mastitis
Klebsiella pneumoniae	4	0.7	mastitis
Listeria monocytogenes	4	0.7	abortion, stillbirth, neurological sign
Moraxella	4	0.7	respiratory sign
Enterobacter (E. cancerogenes, gergoviae)	3	0.5	mastitis
Bacillus cereus	2	0.4	mastitis
Citrobacter freundii	2	0.4	mastitis
Chlamydia pecorum	2	0.4	diarrhea, death
acid-fast bacteria	1	0.2	stillbirth
Acinetobacter baumannii	1	0.2	astasia
Alcaligenes	1	0.2	death, sudden death
Burnholderia cepacia	1	0.2	respiratory sign
Coccobacillus	1	0.2	opacity of eyeball
Gram-negative bacteria	1	0.2	fragility, lameness
Lactococcus lactis	1	0.2	mastitis
Morganella morganii	1	0.2	mastitis
Mycobacterium fortuitum	1	0.2	mastitis

continue to next page

Pathogens	Cases	Ratio (%)	Clinical signs
Prototheca zopfii	1	0.2	mastitis
*Bacteria**	43	7.7	respiratory sign, diarrhea, neurological sign, lameness, astasia, pyrexia, abortion, mass, death, sudden death

* Bacterial species were unknown, but cases in which the involvement of bacteria was histopathologically suspected.

Viruses

Pathogens	Cases	Ratio (%)	Clinical signs
Bovine coronavirus	47	27.6	diarrhea, respiratory sign, death
Bovine respiratory syncytial virus	23	13.5	respiratory sign
Akabane virus	18	10.6	neurological sign, astasia, blindness, stillbirth, malformation
Bovine viral diarrhea virus	13	7.6	diarrhea, respiratory sign, premature birth, death
Rotavirus A	13	7.6	diarrhea, death
Bovine enterovirus	12	7.1	diarrhea
Bovine parainfluenza virus 3	9	5.3	respiratory sign
Bovine adenovirus	8	4.7	diarrhea, respiratory sign, astasia, death
Bovine leukemia virus	8	4.7	mass
Bovine torovirus	6	3.5	diarrhea, respiratory sign, death
Bovine rhinovirus	3	1.8	respiratory sign
Bovine herpesvirus 1	2	1.2	respiratory sign, decrease of lactation yield
Pseudocowpox virus	1	0.6	white vesicles on the tongue surface
Virus*	7	4.1	malformation, astasia, respiratory sign, death

*Viral species were unknown, but cases in which the involvement of virus was histopathologically suspected.

Protozoa

Pathogens	Cases	Ratio (%)	Clinical signs
Eimeria	18	81.8	death, diarrhea
Cryptosporidium parvum	3	13.6	diarrhea
Neospora caninum	1	4.5	stillbirth

Fungi

Pathogens	Cases	Ratio (%)	Clinical signs
Candida	6	40.0	death, diarrhea, mastitis
Aspergillus niger	1	6.7	death, respiratory sign
Absidia	1	6.7	death, respiratory sign
Penicillium	1	6.7	death, respiratory sign
*Fungi**	6	40.0	death, respiratory sign, diarrhea, mastitis

Parasaite

Pathogens	Cases	Ratio (%)	Clinical signs
Fasciola	1	50.0	diarrhea
Strongyloides papillosus	1	50.0	death, sudden death

Table 2-5. Summary of pathogens by use of cattle

Meat calves

Bacteria	Cases	Virus	Cases	Fungi	Cases	Protozoa	Cases	Parasaite	Cases
Mycoplasma	141	Bovine coronavirus	25	*Candida*	5	*Eimeria*	15	*Strongyloides papillosus*	1
Pasteurella	39	Bovine respiratory syncytial virus	11	*Absidia*	1	*Cryptosporidium parvum*	3		
Escherichia coli	23	Bovine enterovirus	10	*Aspergillus niger*	1				
Clostridium	15	Akabane virus	10	*Penicillium*	1				
Ureaplasma diversum	15	Rotavirus A	9	*Fungi**	5				
Mannheimia haemolytica	13	Bovine adenovirus	5						
Trueperella pyogenes	8	Bovine viral diarrhea virus	5						
Pseudomonas	5	Bovine torovirus	4						
Histophilus somni	5	Bovine leukemia virus	1						
Fusobacterium necrophorum	4	Bovine parainfluenza virus 3	1						
Moraxella	4	Bovine rhinovirus	1						
Acinetobacter baumannii	1	Virus*	5						
Burnholderia cepacia	1								
Chlamydia pecorum	1								
Coccobacillus	1								
Gram-negative bacteria	1								
Streptococcus bovis	1								
*Bacteria**	27								

* The species were unknown, but cases in which the involvement of pathogen was histopathologically suspected.

Milk calves

Bacteria	Cases	Virus	Cases	Fungi	Cases	Protozoa	Cases	Parasaite	Cases
Mycoplasma	11	Akabane virus	4			*Eimeria*	2		
Clostridium	7	Bovine coronavirus	3						
Pasteurella multocida	3	Bovine viral diarrhea virus	3						
Listeria monocytogenes	1	Bovine adenovirus	2						
Chlamydia pecorum	1	Bovine enterovirus	1						
Mannheimia haemolytica	1	Rotavirus A	1						
Trueperella pyogenes	1	Virus*	2						
*Bacteria**	6								

* The species were unknown, but cases in which the involvement of pathogen was histopathologically suspected.

Breeding cattle

Bacteria	Cases	Virus	Cases	Fungi	Cases	Protozoa	Cases	Parasaite	Cases
Mycoplasma	6	Bovine leukemia virus	3			*Neospora caninum*	1	*Fasciola*	1
Escherichia coli	3	Bovine viral diarrhea virus	2						
Clostridium	2	Bovine respiratory syncytial virus	2						
Listeria monocytogenes	2	Bovine coronavirus	2						
Pasteurella multocida	2	Rotavirus A	1						
Ureaplasma diversum	2	Pseudocowpox virus	1						
acid-fast bacteria	1	Bovine parainfluenza virus 3	1						
Trueperella pyogenes	1								
*Bacteria**	6								

* The species were unknown, but cases in which the involvement of pathogen was histopathologically suspected.

Dairy cattle

Bacteria	Cases	Virus	Cases	Fungi	Cases	Protozoa	Cases	Parasaite	Cases
Staphylococcus	58	Bovine coronavirus	10	*Candida terebra*	1				
Streptococcus	31	Bovine leukemia virus	3	*Fungi**	1				
Corynebacterium	11	Rotavirus A	2						
Enterococcus	7	Bovine respiratory syncytial virus	2						
Escherichia coli	6	Akabane virus	2						
Serratia marcescens	6	Bovine parainfluenza virus 3	1						
Aerococcus	5	Bovine enterovirus	1						
Klebsiella pneumoniae	4	Bovine herpesvirus 1	1						
Trueperella	4	Bovine viral diarrhea virus	1						
Enterobacter	3	Bovine torovirus	1						
Mycoplasma	3								
Bacillus cereus	2								
Citrobacter freundii	2								
Pseudomonas	2								
Fusobacuterium necrophorum	1								
Histophilus somni	1								
Lactococcus lactis	1								
Listeria monocytogenes	1								
Morganella morganii	1								
Mycobacterium fortuitum	1								
Prototheca zopfii	1								
*Bacteria**	2								

* The species were unknown, but cases in which the involvement of pathogen was histopathologically suspected.

Fattening cattle

Bacteria	Cases	Virus	Cases	Fungi	Cases	Protozoa	Cases	Parasaite	Cases
Mycoplasma	9	Bovine respiratory syncytial virus	8			*Eimeria*	1		
Clostridium	7	Bovine coronavirus	7						
Mannheimia haemolytica	6	Bovine parainfluenza virus 3	6						
Pasteurella multocida	6	Bovine viral diarrhea virus	2						
Escherichia coli	3	Bovine rhinovirus	2						
Histophilus somni	3	Akabane virus	2						
Ureaplasma diversum	3	Bovine torovirus	1						
Fusobacterium necrophorum	2	Bovine leukemia virus	1						
Trueperella pyogenes	2	Bovine herpesvirus 1	1						
Alcaligenes	1	Bovine adenovirus	1						
Corynebacterium striatum	1								
*Bacteria**	2								

* The species were unknown, but cases in which the involvement of pathogen was histopathologically suspected.

Table 2-6. Summary of pathogens by clinical signs

Respiratory sign

Bacteria	Cases	Virus	Cases	Fungi	Cases	Protozoa	Cases	Parasaite	Cases
Mycoplasma	160	Bovine coronavirus	24	*Absidia*	1				
Pasteurella	48	Bovine respiratory syncytial virus	23	*Aspergillus niger*	1				
Mannheimia haemolytica	19	Bovine parainfluenza virus 3	9	*Penicillium*	1				
Ureaplasma diversum	15	Bovine rhinovirus	3	*Fungi**	2				
Trueperella pyogenes	8	Bovine viral diarrhea virus	2						
Escherichia coli	4	Bovine adenovirus	2						
Moraxella	4	Bovine herpesvirus 1	2						
Histophilus somni	4	Bovine torovirus	1						
Pseudomonas	4	Virus*	2						
Clostridium	3								
Fusobacterium necrophorum	2								
Burnholderia cepacia	1								
*Bacteria**	10								

* The species were unknown, but cases in which the involvement of pathogen was histopathologically suspected.

Death

Bacteria	Cases	Virus	Cases	Fungi	Cases	Protozoa	Cases	Parasaite	Cases
Mycoplasma	26	Bovine coronavirus	6	*Candida*	2	*Eimeria*	10	*Strongyloides papillosus*	1
Clostridium	25	Rotavirus A	6	*Absidia*	1	*Cryptosporidium parvum*	2		
Escherichia coli	15	Bovine viral diarrhea virus	4	*Aspergillus niger*	1				
Pasteurella	9	Bovine adenovirus	3	*Penicillium*	1				
Histophilus somni	6	Bovine torovirus	3	*Fungi**	5				
Mannheimia haemolytica	5	Bovine leukemia virus	1						
Trueperella pyogenes	5	Virus*	2						
Pseudomonas aeruginosa	3								
Ureaplasma diversum	3								
Fusobacterium necrophorum	2								
Alcaligenes	1								
Chlamydia pecorum	1								
*Bacteria**	24								

* The species were unknown, but cases in which the involvement of pathogen was histopathologically suspected.

Diarrhea

Bacteria	Cases	Virus	Cases	Fungi	Cases	Protozoa	Cases	Parasaite	Cases
Escherichia coli	12	Bovine coronavirus	27	*Candida*	2	*Eimeria*	17	*Fasciola*	1
Clostridium perfringens	9	Rotavirus A	13	*Fungi**	3	*Cryptosporidium parvum*	2		
Chlamydia pecorum	2	Bovine enterovirus	12						
*Bacteria**	5	Bovine viral diarrhea virus	9						
		Bovine adenovirus	6						
		Bovine torovirus	5						
		Bovine leukemia virus	1						

* The species were unknown, but cases in which the involvement of pathogen was histopathologically suspected.

Mastitis

Bacteria	Cases	Virus	Cases	Fungi	Cases	Protozoa	Cases	Parasaite	Cases
Staphylococcus	58			*Candida terebra*	1				
Streptococcus	29			*Fungi**	1				
Corynebacterium	10								
Enterococcus	7								
Escherichia coli	6								
Serratia marcescens	6								
Aerococcus	5								
Klebsiella pneumoniae	4								
Trueperella	4								
Enterobacter	3								
Bacillus cereus	2								
Citrobacter freundii	2								
Mycoplasma	2								
Pseudomonas	2								
Lactococcus lactis	1								
Morganella morganii	1								
Mycobacterium fortuitum	1								
Prototheca zopfii	1								

* The species were unknown, but cases in which the involvement of pathogen was histopathologically suspected.

3. CHAPTER 2

Application of Real-Time RT-PCR for Diagnosis of Akabane Disease

3.1. ABSTRACT

It is generally difficult to isolate or detect the gene and antigen of Akabane virus (AKAV) from abnormal calves and cattle with neurological signs. In this chapter, real-time RT-PCR (rRT-PCR) was performed to examine the availability for diagnosis, using 10 clinical samples of Akabane disease collected in Yamaguchi Prefecture from September 2011 to January 2012. AKAV genes were mainly detected from the brain stem, spinal cord and cerebellum of nine cattle with neurological signs and one abnormal calf by rRT-PCR. Positive correlations were found among quantity of AKAV gene, the severity of inflammation and intensity of immunohistochemical straining in the central nervous system. These results suggests that rRT-PCR is useful for sensitive and accurate diagnosis of Akabane disease.

3.2. INTRODUCTION

In 2011, encephalomyelitis of cattle by the postnatal infection with Akabane virus (AKAV) frequently occurred in the Chugoku district. In Yamaguchi Prefecture, there were nine postnatal infection cases and one stillbirth case with malformation from September 2011 to January 2012. AKAV genes and antigens were detected in several cattle which showed neurological signs and AKAV was isolated from one case. However, since most cases of Akabane disease are found after virus was cleared from diseased cattle, it is sometimes difficult to confirm the viral infection. Therefore, more sensitive and accurate method is necessary for diagnosis of Akabane disease.

In this Chapter, we applied real-time RT-PCR (rRT-PCR) for diagnosis of Akabane diseases using specimens collected from diseased cattle in Yamaguchi Prefecture. Furthermore, we examined the correlation of results between histopathological examination and rRT-PCR.

3.3. MATERIALS AND METHODS

3.3.1. Cases

Clinical specimens were collected from nine cattle of 3-17 months old (6 Japanese Blacks, 1 crossbred and 2 Holsteins) that showed neurological signs such as astasia, knuckling, hyperesthesia and circling behavior, and one stillbirth Holstein with malformation. The vaccine of Akabane disease was not inoculated for any of those cases.

3.3.2. Detection of AKAV genes from clinical specimens by RT-PCR

Samples of each central nervous system (CNS) were collected from the affected cattle and were homogenized with Eagle's minimum essential medium (MEM). These homogenates were centrifuged at 800 x g for 10 min at 4 °C. The supernatants were filtered through a 450-nm membrane (Merck Millipore, Cork, Ireland) and used for virus isolation and viral RNA extraction. Total RNA was extracted using the High Pure Viral RNA Kit (Roche Diagnostics K.K., Tokyo, Japan). Then, RT-PCR was carried out using one-step RT-PCR kit (Qiagen, Tokyo, Japan) with primers, AKAI172F and AKAI560R, for S segment of Simbu serogroup viruses (SIMV) (Kono *et al*., 2008), AKAVM-F (5'-AAG CAA GAG GAA TGC AGC TCT ACA-3') and AKAVM-R (5'-CTG TTT TGA GGA GTC GAA TAG ACC-3'), AINOVM-F (5'-TGC TAT AGC CCC TTC ATA CAT TGG-3') and AINOVM-R (5'-TGG CAT GTT TGC AGT GGT TAC AGT-3') for M RNA segment of Aino virus, PEAVM-F (5'-CCT TCC ATA CGC CAT TTA GGT GA-3') and PEAVM-R (5'-TGC TCA TCA CAT TCA GAT GA-3') for M RNA segment of Peaton virus. Reverse-transcription was conducted at 50°C for 30 min. This mixture was then heated for 95°C for 15 min to stop the reaction and to activate HotStart Taq DNA polymerase. The resulting cDNA was amplified by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 60 s followed by one step of final extension at 72°C for 10 min. The PCR products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

3.3.3. Virus isolation

Virus isolation was performed by inoculation of homogenate of CNS in hamster lung (HmLu-1) cells. The cells were grown in Eagle's MEM with kanamycin (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.295% tryptose phosphate broth (TPB), 0.015% sodium bicarbonate, 0.03% L-glutamine, and 10% fetal bovine serum at 37 ℃. The cells were cultured in rolling tubes, washed with maintenance medium (MEM containing 0.295% TPB, 0.015% sodium bicarbonate, 0.03% L-glutamine, and 0.1% bovine serum albumin), and inoculated with 0.1 ml of the processed samples. After adsorption for 60 min at 37 ℃, the inoculum was removed and 0.5 ml of maintenance medium was added. The rolling tube-cultured cells were incubated by rotary cultures and observed cytopathic effects (CPE) for at least 7 days. Cell cultures without CPE were passaged twice in a blinded manner.

3.3.4. Sequence analysis

Nucleotide sequences of PCR products of S segment (699 bp) encoding SIMV common antigen (nucleocapsid protein) and M segment (4203 bp) encoding neutralization antigen Gc were determined as described previously (Kobayashi *et al*., 2007).

3.3.5. Phylogenetic analysis

Phylogenetic analyses for S and M segments were carried out based on the previous studies (Kobayashi *et al*., 2007; Yamakawa *et al*., 2006). Phylogenetic trees were constructed using neighbor-joining methods (Saitou *et al*., 1987), and the reliability of the branch was evaluated by bootstrapping with 1,000 replicates.

3.3.6. Dot Blot analysis

To antigenically characterize the isolates, dot blot method (Yoshida *et al*., 1998) was performed using the monoclonal antibody recognizing the N protein, SIMV common antigen, and seven neutralizing epitopes (A1, A2, B, C1, C2, D and E) on the Gc protein of AKAV (Otani *et al*., 2013).

3.3.7. Histopathological examination and IHC

Histopathological examination by the hematoxylin and eosin (HE) staining was performed according to a conventional method. The degree of nonpurulent encephalomyelitis, based on the existence of perivascular cuffing (thickness of infiltrating cells, ratio of small vessel which lesions were formed), glial nodules (size and distribution of nodules), degeneration and necrosis of neurons, and the presence or absence of neuronophagia, were quantified as follows; extremely severe: 4, severe: 3, moderate: 2, mild: 1, very mild: 0.5 and no lesion: 0.

IHC in CNS were performed using anti-AKAV rabbit immune serum and the staining intensity based on positive cells in neuronal cells were quantified as follows; advanced (3), moderate (2), low degree (1), very low degree (0.5), and not detected (0).

3.3.8. rRT-PCR of AKAV

The RNA extracted from CNS was used for rRT-PCR and cDNA was transcribed by a reverse transcription reaction at 25°C for 10 min and 37°C for 30 min using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Kanagawa, Japan). PCR amplifications were carried out with TaqMan Universal PCR Master Mix (Life Technologies Japan Ltd.) using a Applied Biosystems 7500 Real Time PCR System (Thermo Fisher Scientific) for detection of the AKAV S RNA segment (104 bp) with the primer set Forward (5'-TCA ACC AGA AGA AGG CCA AGA T-3'), Reverse (5'-GGG AAA ATG GTT ATT AAC CAC TGT AAA-3') and the TaqMan probe (FAM 5'-GTT GTG GCG TCT TA-3'-NFQMGB) (Yazaki, 2007). The amplification conditions consisted of a preliminary denaturation step of 95 °C for 10 min, 50 cycles of denaturation at 95°C for 15 s, annealing-extension at 60°C for 60 s. The gene dosage of each organ was calculated with positive control designated as value of 1. AKAV JaGAr39 strain (Oya *et al*., 1961) ($10^{6.6}$ $TCID_{50}$/ml) was used for a positive control.

3.3.9. Statistical analysis

To examine correlation among quantity of AKAV, the degree of the lesion and the IHC intensity, the

coefficient of correlation was analyzed by the test of Pearson's correlation coefficient and regression analysis.

3.4. RESULTS

3.4.1. Detection of AKAV genes by RT-PCR and characterization of the isolated virus

AKAV genes were detected in the CNS of all 9 cases except for the stillborn calf by RT-PCR. The genes of AINOV and PEAV were not detected. Two AKAV (YG-1/Br/11 and YG-1/Ce/11 strains) were isolated from brain and cerebellum of 1 case (No. 3).

Nucleotide sequences of S and M segments of these strains were completely determined, resulting that the isolated strains belonged to genogroup 1 by the classification of previous reports (Kobayashi *et al*., 2007; Yamakawa *et al*., 2006). Nucleotide sequences of S segment detected from CNS of nine cattle with neurological signs were identical. Phylogenetic analysis indicated that isolates were the most closely related to strains isolated from cattle with encephalomyelitis in Kyushu in 2006 and in Yamaguchi Prefecture in 2007 (data not shown). Dot blot analysis showed that two isolates were antigenically similar to the Iriki strain (Miyazato *et al*., 1989) which was the representative strain of genogroup 1.

3.4.2. Histopathological characteristics and detection of viral antigens by IHC

Nonpurulent encephalomyelitis was confirmed in nine cattle with neurological signs. The degree of nonpurulent encephalomyelitis was digitized with 0.72, 1.17, 2.56 and 1.80 on the average, for cerebrum, cerebellum, brainstem and spinal cord, respectively, resulting the lesion in brainstem being the severest (Table 3-1). Lesions in the cerebrum were the mildest, and there were cases without inflammation. In the case No. 5 showing abnormal behavior, such as circus movement or the dash, severe lesions were observed in brainstem, cerebellum and cerebrum.

AKAV antigens were detected by IHC in cytoplasm of nerve cells of the brainstem in six of nine cases. Although mild nonpurulent mesencephalitis, the reduction of spinal cord ventral horn nerve cells, and muscular atrophy were confirmed in the stillborn calf (No.10), AKAV antigens were not detected by IHC.

3.4.3. Detection of AKAV by rRT-PCR

In cases of infection after birth, AKAV genes were detected by rRT-PCR as well as RT-PCR. In the stillborn calf, we were not able to detect AKAV by RT-PCR and IHC but could successfully detect AKAV by rRT-PCR.

The gene dosage (geometric mean value) of AKAV in the CNS of cattle infected after birth was the highest in brainstem followed by cerebellum, spinal cord and cerebrum (Table 3-1). The gene dosage of AKAV in the stillborn calf was the highest in spinal cord followed by brainstem and cerebellum, while no detection of AKAV in cerebrum. Gene dosages detected in each CNS organ of the stillborn calf was less than those in cases of infection after birth (Table 3-1)

3.4.4. Correlations among AKAV gene dosage, intensities of lesion and IHC

Significant positive correlations were found between the gene dosage and degree of lesion ($r = 0.702$), the gene dosage and degree of lesion ($r = 0.586$), and the degree of lesion and intensity of IHC ($r = 0.669$) (Figure 3-1).

3.5. Discussion

Since there are few cases of encephalomyelitis by infection with AKAV after birth, the information has not been accumulated yet. AKAV isolated from cerebrum and cerebellum of the calf which presented neurological signs in Yamaguchi Prefecture in 2011 belonged to genogroup 1 genetically, and the antigenically and phylogenetically similar to the strains isolated from cattle with encephalomyelitis in 1984, 2006 and 2007 (Kono *et al*., 2008; Miyazato *et al*., 1989; Otani *et al*., 2008). These data suggest that AKAV genogroup 1 is associated with the development of encephalomyelitis in cattle by the infection after birth.

We confirmed the availability of rRT-PCR using clinical samples of encephalomyelitis by the infection after birth. Furthermore, AKAV genes were detected by rRT-PCR from cattle with negative IHC and calf with negative RT-PCR, indicating that rRT-PCR was the most sensitive method to detect AKAV. Especially, since detection of antibody is the only diagnostic method for AKAV in calf without colostrum, it is valuable that rRT-PCR could detect AKAV form the malformed calf. In addition, since rRT-PCR is less time consuming than the other diagnostic methods, such as IHC and conventional RT-PCR, rRT-PCR must be useful for diagnosis of AKAV.

Kono *et al*. (2008) reported that the intensity of IHC is correlated with the degree of inflammation in Akabane disease. In this study, the correlations were confirmed statistically by digitizing the degree of the lesion and IHC intensity. Moreover, the correlations among the gene dosage and degree of lesion, intensity of IHC were also confirmed by rRT-PCR.

In conclusion, this rRT-PCR method must contribute to the improvement of the diagnosis precision of Akabane disease.

3.6. LEGEND FOR FIGURE

Figure 3-1. Correlations between gene dosage and degree of lesion, gene dosage and IHC intensity and degree of lesion and IHC intensity

Degree of lesion is described as follows. extremely severe (4): extremely severe perivascular cuffing, diffuse glial nodule, denaturation and necrosis of neural cells, neuronophagia. severe (3): severe perivascular cuffing, multiple glial nodules, denaturation and necrosis of neural cells, neuronophagia. moderate (2): moderate perivascular cuffing, sporadic glial nodules. mild (1): mild perivascular cuffing, sporadic glial nodules. very mild (0.5): very mild perivascular cuffing. no lesion (0). IHC intensity is described as follows. high degree (3): positive nerve cells ratio 40 % <. moderate (2): positive nerve cells ratio 10-40 %. low degree (1): positive nerve cells ratio 5-10 %. very low degree (0.5): positive nerve cells ratio 0-5 %. undetected (0).

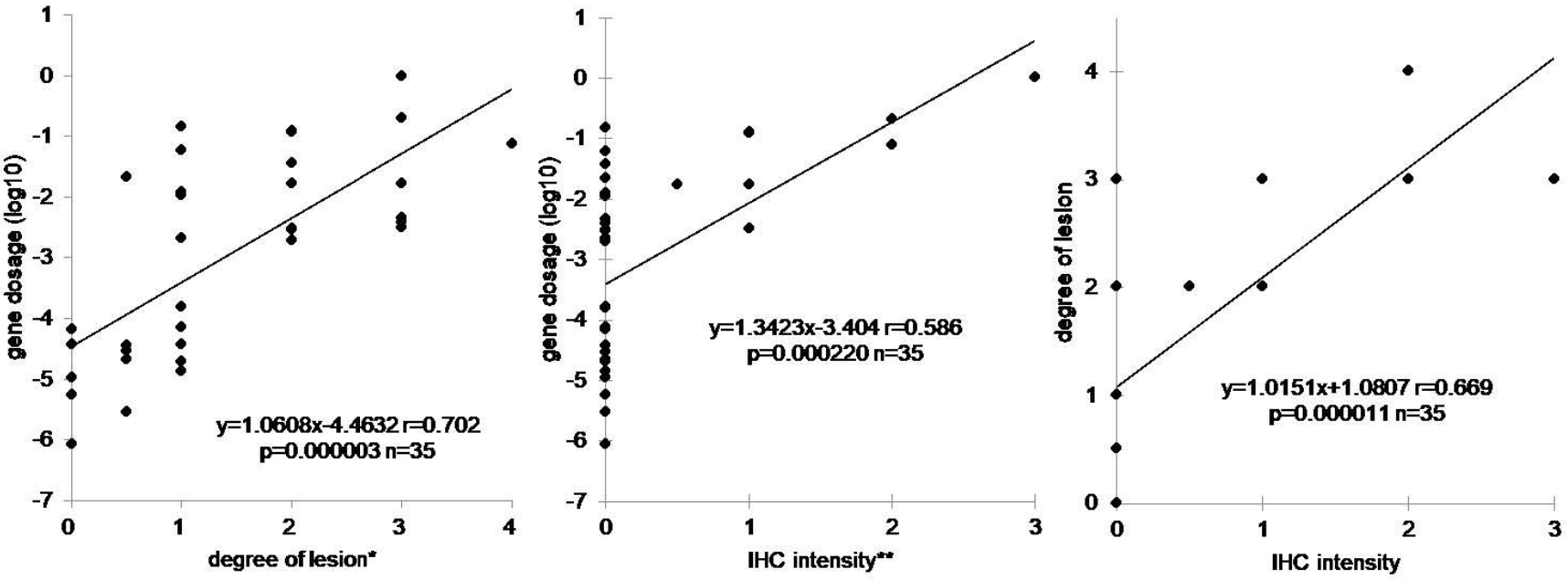

Figure 3-1 Correlations between gene dosage and degree of lesion, gene dosage and IHC intensity, degree of lesion and IHC intensity.

Table 3-1. The degree of lesions, intensity of IHC staining and AKAV gene dosage in CNS

Infection	No.	HE staining				IHC				AKAV gene dosage (log10)			
		Cerebrum	Cerebellum	Brainstem	Spinal cord	Cerebrum	Cerebellum	Brainstem	Spinal cord	Cerebrum	Cerebellum	Brainstem	Spinal cord
After birth	1	1*	1	3		0**	0	2		-1.917	-0.833	-0.693	
	2	0.5	0.5	2	3	0	0	0.5	1	-4.666	-4.536	-1.780	-1.780
	3	1	2	3		0	1	3		-1.975	-0.928	-0.002	
	4	0	1	2	2	0	0	0	0	-4.967	-4.133	-2.714	-2.527
	5	2	3	4		0	0	2		-2.511	-2.336	-1.121	
	6	1	1	3	2	0	0	1	0	-2.668	-1.228	-2.492	-1.433
	7	0	0.5	1	1	0	0	0	0	-4.429	-4.439	-4.860	-4.706
	8	1	1	3	1	0	0	0	0	-3.799	-3.812	-2.407	-4.432
	9	0	0.5	2		0	0	1		-4.173	-1.664	-0.900	
Average		0.7	1.2	2.6	1.8	0	0.1	1.1	0.2	-3.456	-2.657	-1.885	-2.975
Fetus	10	0	0	0.5	0	0	0	0	0	-	-6.063	-5.527	-5.253

*degree of lesion
Extremely severe (4): extremely severe perivascular cuffing, diffuse glial nodule, denaturation and necrosis of neural cells, neuronophagia
Severe (3): severe perivascular cuffing, multiple glial nodules, denaturation and necrosis of neural cells, neuronophagia
Moderate (2): moderate perivascular cuffing, sporadic glial nodules
Mild (1): mild perivascular cuffing, sporadic glial nodules
Very mild (0.5): very mild perivascular cuffing
No lesion: (0)
** IHC intensity
High degree (3): positive nerve cells ratio 40 % <
Moderate (2): positive nerve cells ratio 10-40 %
Low degree (1): positive nerve cells ratio 5-10 %
Very low degree (0.5): positive nerve cells ratio 0-5 %
Undetected (0)
Blank: no specimen

4. CHAPTER 3

Genetic and antigenic analysis of *Chlamydia pecorum* strains isolated from calves with diarrhea

4.1. ABSTRACT

Chlamydia pecorum (designated 22–58) was isolated in 2010 in HmLu-1 cells from the jejunum of a calf which died of necrotizing enterocolitis in Yamaguchi Prefecture, Japan. Immunohistochemical staining identified *C. pecorum* positive reactions in the jejunal villi. *C. pecorum*, designated 24–100, was isolated from the feces of a calf with diarrhea in another farm in Yamaguchi Prefecture in 2012. A significant increase in neutralizing antibody titers against *C. pecorum* was confirmed in paired sera. Nucleotide sequence identities of *omp1* genes of the 2 isolates were 100%. The isolates were genetically and antigenically more closely related to *C. pecorum* Bo/Yokohama strain isolated from cattle with enteritis in Japan than to the other prototype strains, Bo/Maeda isolated from cattle with pneumonia and Ov/IPA isolated from sheep with polyarthritis. These results indicate that *C. pecorum* strains similar to 22–58 and 24–100 might be endemic in Yamaguchi Prefecture and cause enteric disease in cattle.

4.2. INTRODUCTION

Chlamydiaceae are obligate intracellular bacteria that have a unique developmental cycle which includes morphological changes. They cause a wide range of diseases in animals and humans (Storz 1988; Storz *et al*., 1993). The most important mode of transmission is thought to be fecal shedding by carrier animals. Chlamydiae may be shed in vaginal, ocular and nasal discharges, uterine fluid, placental tissue, urine and semen. Chlamydial infection can be acquired by direct contact between animals or by indirect transmission, such as the fecal-oral route or via inhalation of contaminated air (Reinhold *et al*., 2011). Clarkson *et al*. (1997) reported that *Chlamydia* was isolated from lamb's feces on 26 farms in England and Wales, and the prevalence of infection varied from 5–50% on individual farms. Isolates were identified as enteric type rather than abortion type and thus were classified as *Chlamydia pecorum* rather than *Chlamydia psittaci*. In cattle, *C. pecorum* is known to cause encephalomyelitis, pneumonia and enteritis, but subclinical and persistent infections are more common (Fukushi *et al.*, 1995). DeGraves *et al*. (2003) reported that low-level of *C. psittaci* and *C. pecorum* genital infections was detected in 53% of virgin heifers by quantitative PCR, suggesting predominantly extra-genital transmission of *Chlamydia* in cattle. Reinhold *et al*. (2008) reported that natural infections with *Chlamydia* spp. in calves were associated with subclinical chronic effects on animal health. Recently, it was reported that asymptomatic endemic *C. pecorum* infections reduced growth rates in calves by up to 48% (Poudel *et al*., 2012). However, the pathogenesis of *C. pecorum* is still unclear. In Japan, there are only a few reports of *C. pecorum* strains isolated from affected cattle (Fukushi *et al*., 1988, 1989, 1995).

In 2010 and 2012, two *C. pecorum* strains were isolated from cattle with enteritis in Yamaguchi Prefecture and were genetically and antigenically characterized.

4.3. MATERIALS AND METHODS

4.3.1. Clinical signs in diseased calves

Case 1: In May 2010, a Holstein calf (female, 84 days old) showed anorexia, pyrexia and diarrhea in a depositary raising farm in Yamaguchi Prefecture. Despite treatment with antibiotics (kanamycin and tylosin), sulfamonomethoxine and antipyretic, the calf died 20 days after the onset of diarrhea. Case 2: In July 2012, a Japanese black calf (female, 246 days old) on another farm in Yamaguchi Prefecture repeated diarrhea despite treatment with antibiotics (kanamycin) and a probiotic product after the birth, and then, feces were collected during the acute phase for diagnosis. Paired serum samples were collected during the acute and convalescent phases.

4.3.2. Isolation of microorganisms

Tissue specimens obtained from major organs including cerebrum, jejunum, ileum, cecum, colon and ideal contents of the dead calf (case 1) and feces of the second calf (case 2) were minced and homogenized in serum-free Eagle's minimum essential medium with kanamycin (MEM) (Nissui Pharmaceutical Co., Tokyo, Japan). After centrifugation of the 10% homogenate, the supernatant was filtered through a 450 nm membrane (Merck Millipore Ltd., Carrigtwohill, Ireland), and 0.15 ml of the supernatants were inoculated onto hamster lung (HmLu-1) cells, Mardin-Darby bovine kidney (MDBK) cells, bovine testicular (BT) cells, human rectal adenocarcinoma (HRT-18) cells and Vero cells in 24-well plates. After adsorption for 60 min at 37°C, the cells were washed with MEM, and then, 0.5 ml of MEM containing 0.1% bovine serum albumin (Bovogen Biologicals, Williams Avenue, Australia) was added to each well. The cells were incubated at 37°C, and cytopathic effects (CPE) were observed. After incubation for 10 days, cells were frozen and thawed once and then centrifuged. Subsequent passages were carried out at least twice in the same manner with 0.15 ml of the supernatant. Gimenez stain was used to identify CPE. DHL Agar, Columbia agar with 5% sheep blood and

GAM agar with 5% yolk and 0.1% cysteine were used for bacterial isolation. The gene of *Clostridium perfringens* toxin isolated from small-intestinal contents was identified by PCR (Baums *et al*., 2004).

4.3.3. Identification of isolates by PCR, sequence and phylogenetic analysis of *C. pecorum omp1* gene

C. pecorum-infected cells and the supernatants from organs described above were used for DNA extraction. Total DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN, Hiden, Germany). PCR was carried out with TaKaRa Ex Taq Hot Start Version (TaKaRa Bio Inc., Otsu, Japan). Primer pairs targeting fragments of genus-specific and species-specific Chlamydia *omp*1 genes were used for PCR as described by Kaltenböck *et al*. (1997). PCR products were electrophoresed on 2.0% agarose gel and visualized using ethidium bromide staining. PCR products were purified using MiniElute PCR Purification Kit (QIAGEN, Germantown, MD, U.S.A.) and directly sequenced using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Austin, TX, U.S.A.). Phylogenetic analysis was performed using the MEGA 5 program. Sequence data were aligned using ClustalW method (Thompson *et al*., 1994). Genetic distances were calculated using the Tamura-Nei model (Tamura *et al*., 1993). Phylogenetic trees were constructed using neighbor-joining methods (Saitou *et al*., 1987), and the reliability of the branch was evaluated by bootstrapping with 1,000 replicates.

4.3.4. Antigenic analysis of *C. pecorum* isolates

The antigenicity of isolates was compared by immunoblot analysis using rabbit antisera to Bo/Yokohama, Bo/Maeda and Ov/IPA strains of *C. pecorum* (Fukushi *et al*., 1988, 1989). Antisera to Bo/Maeda and Ov/IPA strains were prepared as described by Fukushi and Hirai (Fukushi *et al*., 1988). HmLu-1 cells were infected with *C. pecorum* 22–58, Bo/Yokohama, Bo/Maeda and Ov/IPA strains and *C. psittaci* Cal10 (Francis *et al*., 1938) strain and then incubated at 37°C in 5% CO_2 until CPE was observed. The cells were

recovered from dishes with 0.02% EDTA in phosphate-buffered saline (PBS). After centrifugation at 200 × g for 5 min at 4°C, the supernatant was removed, and the cells were resuspended in PBS. Cells were then mixed with an equal volume of 2 × concentrated sample buffer consisting of 6.25 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol and 0.001% bromophenol blue. Samples were boiled for 3 min, placed on ice for 3 min and centrifuged at 13000 x g for 3 min at room temperature. Cell lysates were electrophoresed using 12% SDS-PAGE and transferred to a PVDF membrane (Immobilon-P; Millipore, Billerica, MA, U.S.A.). After blocking with Tris-buffered saline (TBS) containing 3% gelatin (EIA Grade Reagent Gelatin; Bio-Rad, CA, U.S.A.) for 45 min at 37°C, the membrane was washed three times with TBS containing 0.05% Tween 20 (T-TBS). After incubation with diluted rabbit antisera for 45 min at 37°C, the membrane was washed 3 times with T-TBS. Then, the membrane was reacted with peroxidase-conjugated purified recombinant protein A/G (Thermo Fischer Scientific, Rockford, IL, U.S.A.) for 45 min at 37°C. After washing the membrane with T-TBS and TBS three times each, specific bands were visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Rabbit antisera described above were used for the cross-neutralization test. Sera were serially diluted twofold in MEM in 96-well microplates. Each dilution was mixed with an equal volume of 200 $TCID_{50}$/0.05 ml of *C. pecorum* strains and incubated at 37°C for 1 hr. Then, 0.1 ml (approximately 6×10^3 cells) of HmLu-1 cells in MEM containing 10% fetal bovine serum was added to each well. After incubation at 37°C for 7 days, antibody titers were expressed as the reciprocal of the highest dilution of serum which inhibited CPE completely.

4.3.5. Histological and IHC analyses

Tissue specimens obtained from major organs, intestines and central nervous system of the dead calf (case 1) were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Paraffin-embedded tissues were cut at 2 μm and stained with hematoxylin-eosin. The sections were also used for immunohistochemical

detection of *C. pecorum* antigens. After deparaffinization and rehydration of the paraffin sections, endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 for 10 min. Sections were incubated with 10% normal goat serum for 30 min and then with a rabbit anti-*C. pecorum* Bo/Yokohama strain antiserum (Fukushi *et al*., 1988) (1:2000) for 30 min. Sections were rinsed three times in PBS containing 0.2% Tween 20 for 10 min and then incubated with Histofine Simple Stain MAX-PO (R) (Nichirei Biosciences, Inc., Tokyo, Japan) for 30 min. The sections were rinsed three times in PBS containing 0.2% Tween 20 for 10 min. The sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride under the microscope to control color development, washed in tap water for 10 min and rinsed with distilled water. The sections were counterstained with Meyer's hematoxylin.

4.4. RESULTS

4.4.1. Isolation of microorganisms and PCR for virus detection

Characteristic CPE appeared in HmLu-1 cells inoculated with jejunum homogenate from case 1 (22–58 strain) at the first passage (Figure 4-1). CPE was observed seven days after inoculation. On the other hand, at the second passage, CPE similar to that of 22–58 strain appeared in HmLu-1 cells inoculated with calf feces from case 2 (24–100 strain). Gimenez staining of HmLu-1 cells infected with these strains revealed many small purplish-red particles. *C. pecorum*-specific genes were detected from two isolates. In addition, *C. pecorum*-specific genes were detected from jejunum, ileum, cecum and colon of case 1. *Clostridium perfringens* was isolated from jejunal and ileal contents of case 1 at approximately 10^5 CFU/g. The gene of α-toxins was shown in these isolates by PCR, and isolates were identified as type A (data not shown).

No other viruses were isolated from specimens, and PCR was also negative for all specimens from cases 1 and 2 when tested for bovine viral diarrhea virus (Vilček *et al*., 1994), bovine coronavirus (Tsunemitsu *et al*., 1999), rotavirus (Chinsangaram *et al*., 1994; Gouvea *et al*., 1990; Tsunemitsu *et al*., 1996), bovine torovirus (Hoet *et al*., 2003), bovine enterovirus (Jiménez-Clavero *et al*., 2005), paramyxoviruses (Tong *et al*., 2008), bovine adenovirus (Maluquer de Motes *et al*., 2004) and bovine herpesvirus 4 (Asano *et al*., 2003).

4.4.2. Identification of isolates by PCR, sequence and phylogenetic analysis

Two isolates (22–58 and 24–100) were PCR positive when genus-specific and species-specific *omp1* genes of *C. pecorum* were targeted. Nucleotide sequences of genus-specific PCR products were determined and compared with those of published *C. pecorum omp1* genes. *Omp1* gene partial sequence of the 2 isolates was 100% identical and showed the highest identity (99.8%) to the Bo/Yokohama strain isolated from cattle with enteritis in Japan. Nucleotide similarities of the *omp1* gene between the isolates and other published *C. pecorum* isolates were 89.2%, 85.2% and 82.0% for Bo/Maeda strain which was isolated from cattle with

pneumonia in Japan, Ov/IPA strain which was isolated from sheep with polyarthritis in United States, and 66P130 strain which was isolated from cattle with enteritis in United States, respectively (Figure 4-2, Table 4-1).

4.4.3. Antigenic characterization of *C. pecorum* isolates

The 38 to 40 kDa major outer membrane protein (Fukushi *et al*., 1988) of 22–58 strain strongly reacted with rabbit antiserum raised against the Bo/Yokohama strain. On the other hand, no specific reaction was detected by immunoblot analysis with rabbit antisera to Bo/Maeda or Ov/IPA strains (Figure 4-3). Antigenicity between isolates (22–58 and 24–100) and the 3 strains (Bo/Yokohama, Bo/Maeda and Ov/IPA) was compared using cross-neutralization tests with rabbit antisera. Isolates 22–58 and 24–100 were antigenically similar to the Bo/Yokohama strain, but were a little different from the Bo/Maeda and Ov/IPA strains (Table 4-2).

4.4.4. Neutralization test using paired sera of case 2

A significant increase in antibody against 24–100 strain was confirmed by neutralizing tests using paired sera. Neutralizing titers in sera collected during the acute phase of infection were less than 1:2, but increased to 1:4 during the convalescent phase.

4.4.5. Histopathology and IHC analyses for *C. pecorum* antigens in tissues from case 1

Systemic hyperemia and congestion together with necrosis of villi with short or long rod-shaped bacteria in the upper part of the small intestine were observed in tissue sections. Hemorrhagic ileitis with edematous and granulomatous changes was observed in the intestinal crypts of the ileum. Hemorrhagic colitis was observed in the cecum and colon. Multiple hemorrhages were observed in the mesenteric lymph nodes. In

some organs, historrhexis and decreasing staining properties due to postmortem changes were observed. Positive reactions were observed in the jejunal villi by IHC with rabbit anti-*C. pecorum* antiserum (Figure 4-4).

4.5. DISCUSSION

In this study, *C. pecorum* was isolated from specimens obtained from 2 calves with diarrhea. In case 1, the bacterium was isolated from jejunum, and *C. pecorum* specific *omp1* genes were detected from several locations in the intestines. Additionally, *C. pecorum* antigens were observed in the jejunal villi by immunohistochemical staining. Necrotizing enterocolitis due to *Clostridium perfringens* infection was also observed. Therefore, we speculated that *C. pecorum* might exacerbate the disease caused by *Clostridium perfringens*.

In these 2 cases, it is possible that *C. pecorum* was either the primary pathogen or an exacerbating factor causing diarrhea. On the other hand, it is thought that *C. pecorum* causes asymptomatic infections (Fukushi *et al*., 1995). Recently, Poudel *et al*. (2012) reported that asymptomatic endemic *C. pecorum* infections reduce growth rates in calves by up to 48%. They considered that the mechanism of growth suppression by subclinical chlamydial infection was malabsorption of nutrients due to a local inflammatory response to intestinal mucosal infection. Additionally, despite the absence of clinical signs, chlamydial infection was associated with reduced serum iron concentrations and lower hematocrit values, and infected calves were leukopenic (Reinhold *et al*., 2008). Mohamad and Rodolakis (2010) reported that the persistence of *C. pecorum* strains in the intestines and vaginal mucus of ruminants could cause longterm sub-clinical infection which may affect the animal's health. This may explain the poor weight gain observed in case 2 after recovery from diarrhea. Further studies on the pathogenesis of *C. pecorum* infections are required.

Although the standard method for detecting antibodies to *Chlamydiaceae* spp. in animals is still the complement fixation test (Reinhold et al., 2011), our study showed that a neutralization test was also a useful method for diagnosis of *C. pecorum* infections. In addition, in the dead calf, it was confirmed that immunohistochemistry for *C. pecorum* antigens was also useful.

Genetic and antigenic analysis showed that 22–58 and 24–100 strains were more closely related to

Bo/Yokohama strain isolated from cattle with enteritis than to Bo/Maeda strain isolated from cattle with pneumonia and Ov/IPA strain isolated from sheep with polyarthritis. Bo/Yokohama-like *C. pecorum* strains might cause enteritis more than other serotypes. However, the 2 isolates showed higher sequence identities to Bo/Maeda and Ov/IPA strains than to 66P130 strain isolated from cattle with enteritis in United States. Thus, sequence identities of *C. pecorum omp1* gene might vary according to their geographical background.

The isolation of similar strains from different locations in separate years suggests that *C. pecorum* might be spreading among the cattle population in Yamaguchi Prefecture. An epidemiological study of *C. pecorum* is being conducted currently to clarify the seroprevalence and relationship with disease.

In conclusion, this study showed that *C. pecorum* isolates similar to Bo/Yokohama might be endemic in Yamaguchi Prefecture and cause enteric diseases in cattle.

4.6. LEGEND FOR FIGURES

Figure 4-1. Characteristic CPE observed in Hmlu-1 cells inoculated with homogenized jejunum supernatant from case 1.

Figure 4-2. Phylogenetic tree of *C. pecorum* strains based on the nucleotide sequence of the *omp1* gene (534 nucleotides). The sequences of reference strains were obtained from GenBank. GenBank accession number are L71 (AF269280), LW613 (AJ440240), SBE (EU684916), AB10 (EU684917), M14 (EU684920), 824 (EU684922), BE53 (EU684923), iB1 (EU684924), iB2 (EU684925), iB3 (EU684926), iB4 (EU684927), iB5 (EU684928), W73 (EU684929), R69 (EU684930), iC3 (EU684932), iC4 (EU684933), 1710S (GQ228167), 1920BRZ (GQ228168), 66P130 (GQ228180), 1708 (GQ228194) and MC MarsBar UGT (HQ457473). The nucleotide sequence of *C. psittaci* was used as an outgroup to root the tree. The percentage bootstrap values calculated from 1000 replications are indicated above the internal nodes.

Figure 4-3. Immunoblot analysis of isolates using rabbit antiserum against *C. pecorum*. Antiserum against Bo/Yokohama (A), Bo/Maeda (B), and Ov/IPA (C) strains were used for each figure. Lane 1: mock-infected HmLu-1 cells; Lane 2, 22-58 strain-infected HmLu-1 cells; Lane 3, Bo/Maeda strain-infected HmLu-1 cells; Lane 4, Bo/Yokohama strain-infected HmLu-1 cells; Lane 5, Ov/IPA strain-infected HmLu-1 cells; Lane 6, *C. psittaci*-infected HmLu-1 cells. Arrows indicate a specific band of major outer membrane protein (38 - 40kD).

Figure 4-4. Detection of *C. pecorum* antigens in the jejunum. *C. pecorum*-positive reactions are observed in the jejunal villi (arrows). Immunohistochemistry. Bar = 100 μm

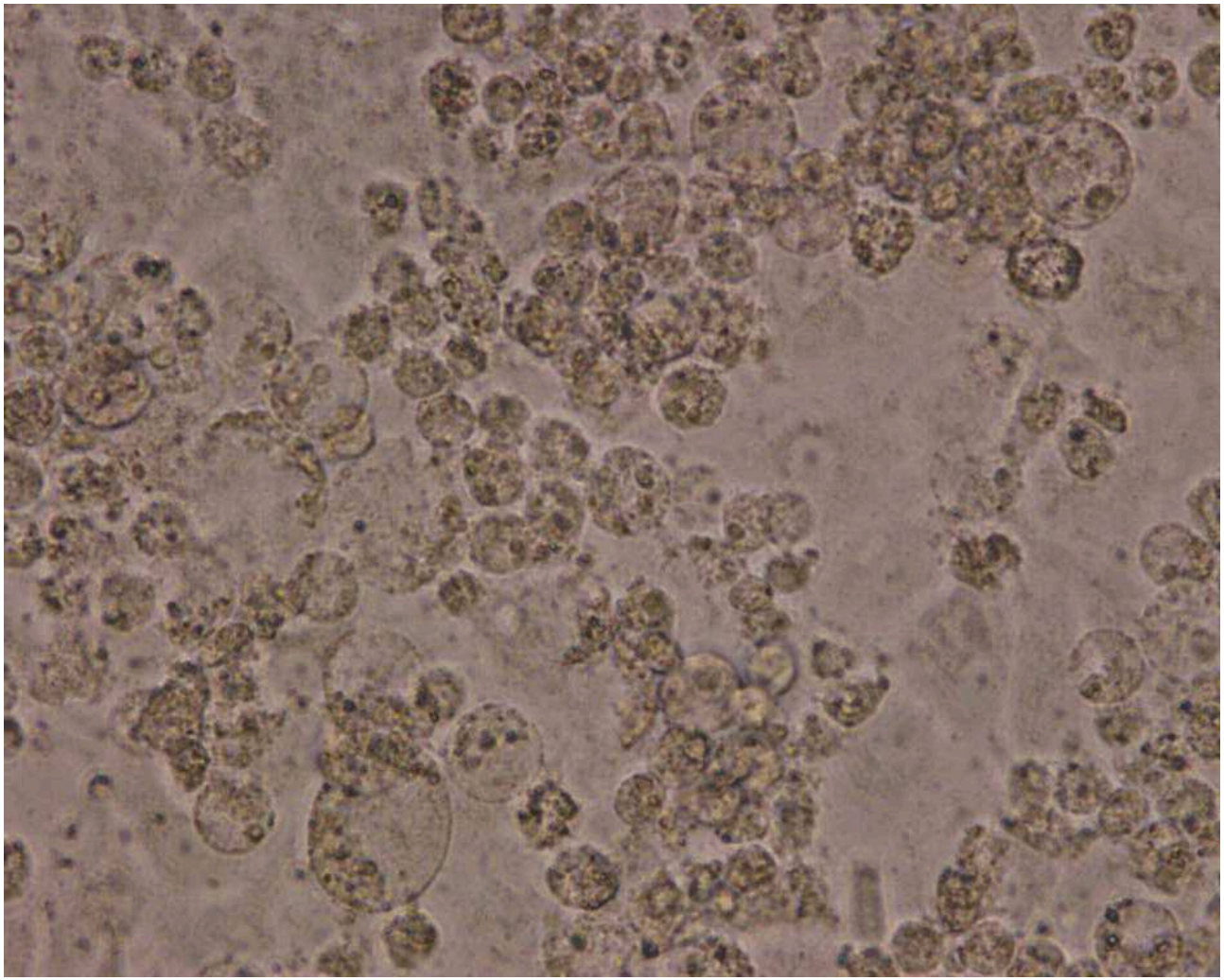

Figure 4-1. Characteristic CPE observed in Hmlu-1 cells inoculated with homogenized jejunum supernatant from case 1.

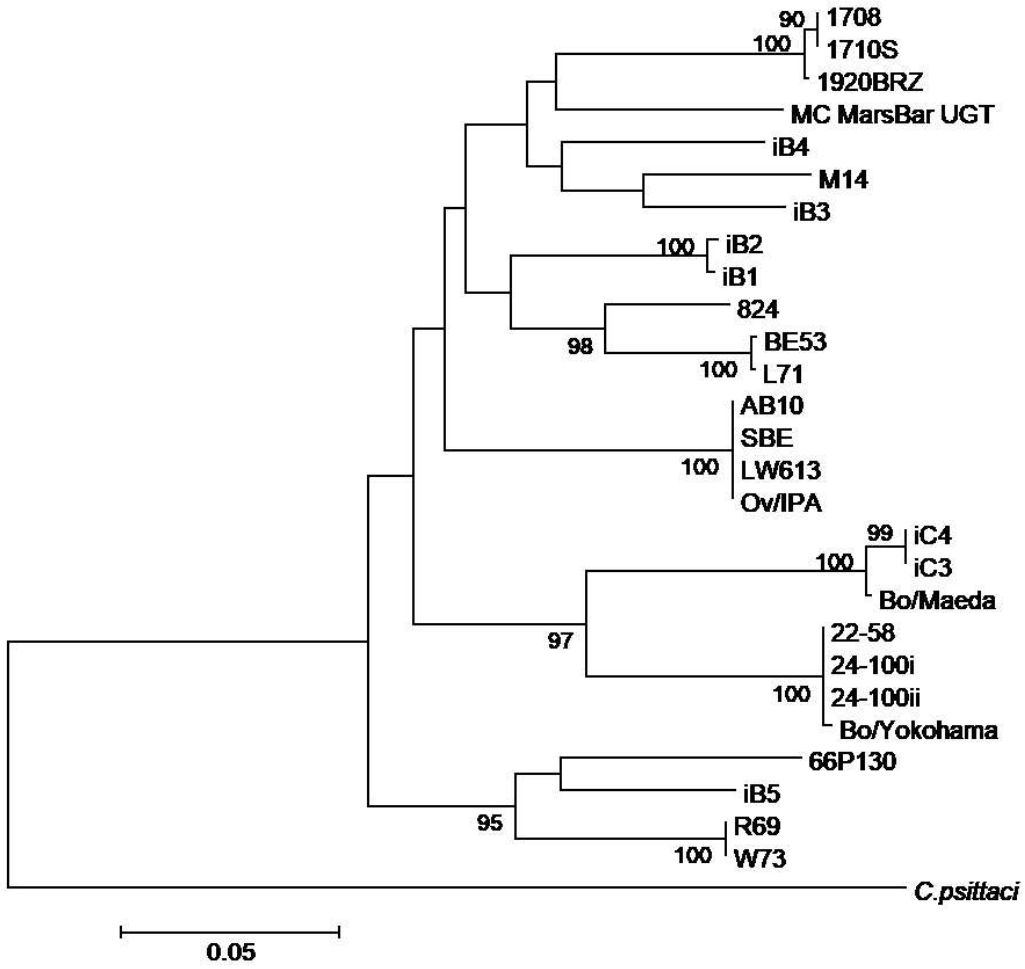

Figure 4-2. Phylogenetic tree of *C. pecorum* strains based on the nucleotide sequence of the *omp1* gene (534 nucleotides).

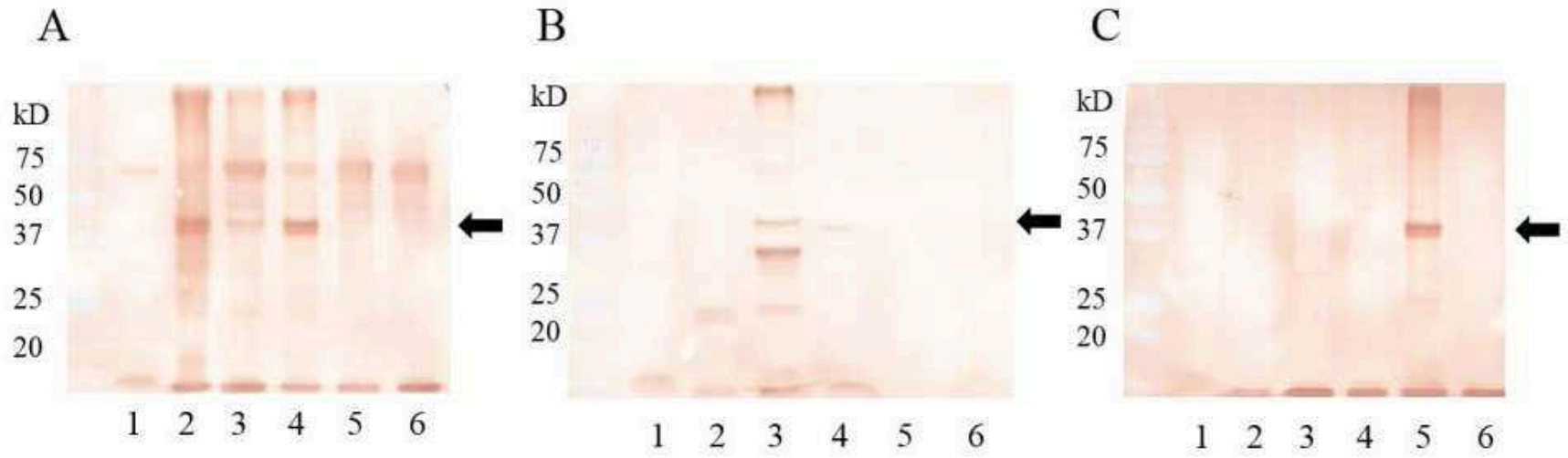

Figure 4-3. Immunoblot analysis of isolates using rabbit antiserum against *C. pecorum*.

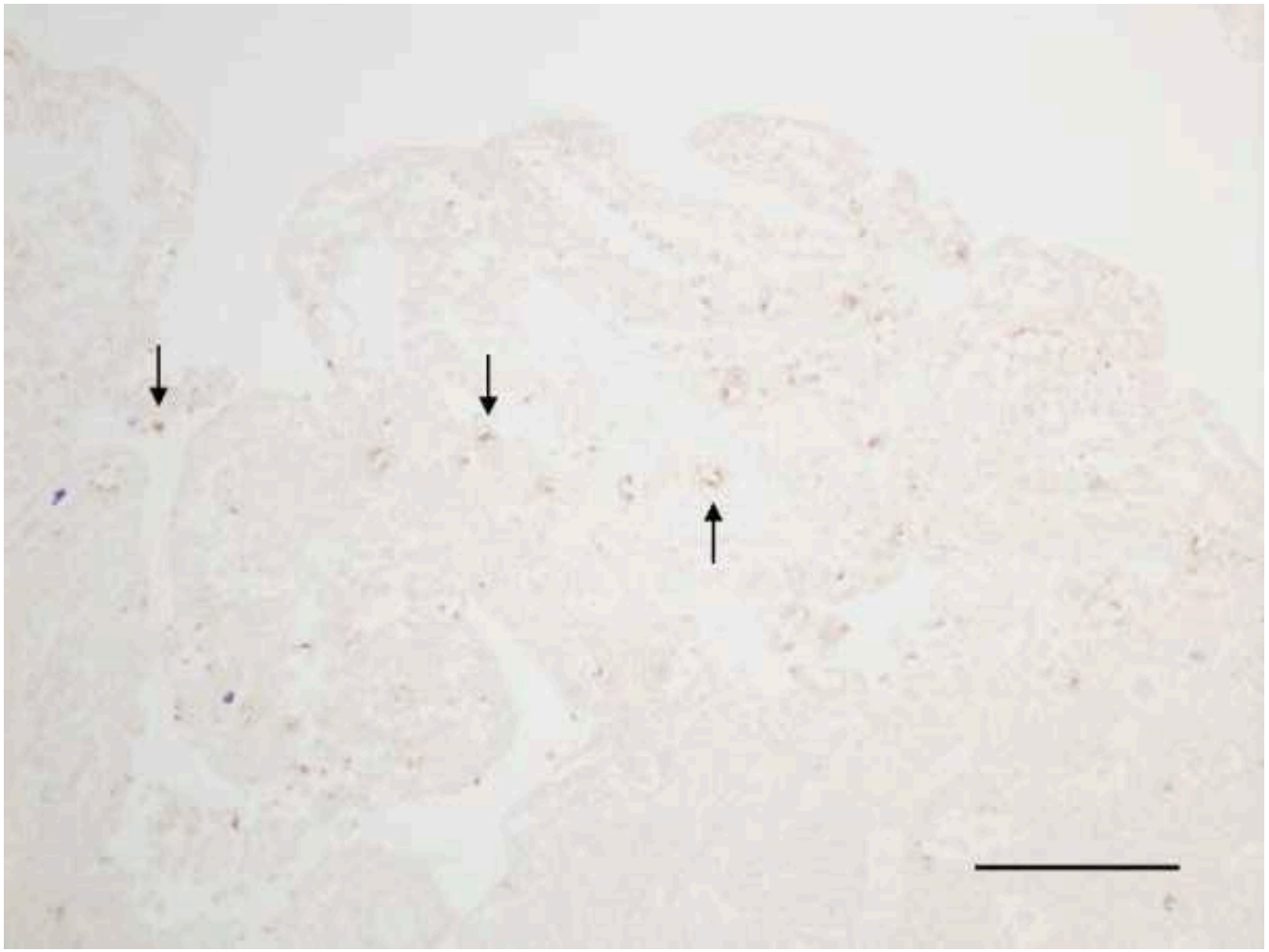

Figure 4-4. Detection of *C. pecorum* antigens in the jejunum.

Table 4-1. Nucleotide sequence identities (%) of *omp1* genes among *C. pecorum* strains.

Strain	22-58	24-100	Bo/Yokohama	Bo/Maeda	Ov/IPA	66P130
22-58	-	100.0	99.8	89.2	85.2	82.0
24-100		-	99.8	89.2	85.2	82.0
Bo/Yokohama			-	89.0	85.0	81.8
Bo/Maeda				-	84.2	83.0
Ov/IPA					-	80.2
66P130						-

Table 4-2. Cross-reactivity between *C. pecorum* strains determined by neutralization test.

Strain	Antibody titer to antisera against		
	Bo/Yokohama	Bo/Maeda	Ov/IPA
22-58	8[a)]	<2	<2
24-100	4	<2	<2
Bo/Yokohama	16	4	<2
Bo/Maeda	4	8	<2
Ov/IPA	<2	<2	4

a) The reciprocal of the highest dilution of serum which inhibited CPE completely.

5. CHAPTER 4

First isolation and genetic characterization of pseudocowpox virus from cattle in Japan

5.1. ABSTRACT

Pseudocowpox virus (PCPV) is endemic among cattle worldwide with zoonotic potential but has not been isolated in Japan. Thus, the epidemiological status of PCPV infection in cattle is undetermined. In May 2016, cattle in a farm in Yamaguchi Prefecture showed white vesicles and hyperemia on the mucosa under the tongue surface, but not on the teats and coronary cushions. A parapoxvirus was isolated from the oral lesion swab and was genetically characterized based on the full-length sequence of *B2L* gene encoding viral envelope. Phylogenetic analysis showed that the isolated virus was classified into PCPV. This is the first report of isolation of PCPV in Japan.

5.2. INTRODUCTION

Pseudocowpox virus (PCPV) is a member of the genus *Parapoxvirus* in the family *Poxviridae*, which includes bovine papular stomatitis virus (BPSV) and orf virus (ORFV) (Knowles, 2011). Parapoxviruses are commonly known as causative agents of dermal diseases in ruminants worldwide, leading to papular stomatitis and contagious pustular dermatitis, especially in the regions of the lips, nostrils, oral mucosa, and teats. The importance of PCPV is increasingly recognized, primarily because of economic losses to farmers in connection with disease outbreaks and because of their zoonotic potential (Friederichs *et al*., 2014).

In Japan, although serological surveys have revealed that seroprevalence of parapoxvirus is very high in cattle and sheep (Kuroda *et al*., 1999; Sentsui *et al*., 2000) and multiple BPSVs have been isolated (Inoshima *et al*., 2001), no PCPV have been isolated yet. Thus, the epidemiological status of PCPV infection in cattle is undetermined.

We here report the first case of the isolation of PCPV in Japan. We determined the full-length sequence of the *B2L* gene encoding viral envelope of this isolate and evaluated its phylogenetic relation to known members of this virus group.

5.3. MATERIALS AND METHODS

5.3.1. Clinical and epidemiological investigations

In May 2016, a breeding cow (Japanese Black, female, 13-month old) in a farm in Yamaguchi Prefecture, in the western part of Japan, showed anorexia, mild fever, frothy salivation, and hyperemia in the mucosa under the tongue surface. No lesions were observed on the teats or coronary cushions. A few days after the onset of clinical signs, the cattle showed white vesicles on the tongue surface (Figure 5-1). These signs were convalescent in about 1 week. No other cattle in the herd showed clinical signs.

5.3.2. Sample collection

An oral swab sample was collected from the mucosal lesions of the affected cattle and was homogenized with Eagle's minimum essential medium (MEM). The sample was centrifuged at 800 x *g* for 10 min at 4°C. The supernatant was filtered through a 450-nm membrane (Merck Millipore, Cork, Ireland) and used for virus isolation and DNA extraction.

5.3.3. Virus isolation

Virus isolation was performed by inoculating homogenates on to the primary bovine testis (BT) cells; two cell lines, hamster lung (HmLu-1) and Madin-Darby bovine kidney (MDBK) cells were also tested for comparison. The cells were grown in Eagle's MEM with kanamycin (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.295% tryptose phosphate broth (TPB), 0.015% sodium bicarbonate, 0.03% l-glutamine, and 5–10% fetal bovine serum at 37°C. The cells were cultured in rolling tubes and 24-well plates, washed with maintenance medium (MEM containing 0.295% TPB, 0.015% sodium bicarbonate, 0.03% l-glutamine, and 0.1% bovine serum albumin), and inoculated with 0.1 ml of the processed samples. After adsorption for 60 min at 37°C, the inoculum was replaced with 0.5 ml of maintenance medium. The rolling tube-cultured and 24-well

plates-cultured cells were incubated by rotary cultures and stationary cultures, respectively, and observed daily for cytopathic effects (CPE) for at least seven days. Cell cultures without CPE were passaged twice in a blinded manner.

5.3.4. Genetic analysis of PCPV

DNA was extracted both from the original oral swab sample and the BT cells showing a CPE by using magLEAD 12gC (Precision System Science, Chiba, Japan). Polymerase chain reaction (PCR) amplifications were carried out with TaKaRa Ex Taq Hot Start Version (TaKaRa Bio, Shiga, Japan) using a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Kanagawa, Japan) for detection of the full-length (1137bp) and partial-length (554bp) *B2L* gene encoding envelope of parapoxvirus with the primer sets OVB2LF1/OVB2LR1 (Hosamani *et al*., 2006) and PPV1/PPV4 (Inoshima *et al*., 2001), respectively. PCR products were purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany), and the nucleotide sequence was determined by direct sequencing using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Austin, TX, U.S.A.). Sequence data were aligned using the ClustalW method (Thompson *et al*., 1994). Phylogenetic analysis was performed using MEGA 6 software (Tamura *et al*., 2013). Phylogenetic trees were constructed using maximum-likelihood methods, and the reliability of the branches was evaluated by bootstrapping with 1000 replicates. Nucleotide and deduced amino acid sequences were compared with those of available corresponding parapoxviruses (Table 5-1).

5.4. RESULTS

At the third passage, a distinct CPE was observed from 2 to 3 days after inoculation in the rolling tube-cultured BT cells, characterized by a rounded morphology and cell detachment (Figure 5-2), while no CPE appeared in the other cell lines or in stationary cultures after third passages. We designated the isolate as strain YG2828. For histological observations, confluent monolayers of BT cells in the chamber slide system were inoculated with the isolate. Twenty-four hours after inoculation, the BT cells were fixed with acetone and stained with hematoxylin-eosin, which revealed pyknosis, and eosinophilic and basophilic cytoplasmic inclusion bodies (data not shown).

Fragments of expected size were amplified by PCR using both primer sets, targeting the full-length (1137bp) and partial-length (554bp) *B2L* gene encoding envelope of parapoxvirus. Neither deletions nor insertions in the nucleotide sequence of the YG2828 strain were found (Figure 5-3). Based on the nucleotide/amino acid identities and phylogenetic analysis of the full-length *B2L* gene, the YG2828 strain was classified as PCPV (Figure 5-4). The nucleotide identities against published parapoxviruses ranged from 85.8% to 98.6% (Table 5-1) and showed the highest identity (98.6%) to three PCPV strains; F05.990C and F10.3081C isolated from cattle in 2005 and 2010 in Finland, respectively, and VR634 isolated from a human in the USA in 1963 with “milker’s nodules” on the hands. The deduced amino acid identities ranged from 84.4% to 99.2% and showed the highest identity (99.2%) to the VR634 strain (Table 5-1), even though these strains were isolated independently as chronologically and geographically. From these samples, Bovine viral diarrhea virus (Vilček *et al*., 1994), epizootic hemorrhagic disease virus (Ohashi *et al*., 2004), bluetongue virus (Ohashi *et al*., 2004), ovine herpesvirus 2 (Baxter *et al*., 1993) and bovine herpesvirus 1 (Rocha *et al*., 1998) were not detected by PCR using specific primers for detection of each viruses (data not shown).

5.5. DISCUSSION

As described above, seroprevalence of parapoxvirus is very high in cattle in Japan and multiple BPSVs have been isolated (Inoshima *et al*., 2001), but no PCPV has been isolated yet. In this study, a PCPV was firstly isolated in Japan by rotary cultures, but not stationary cultures. Similarly, Mavromoustakis *et al*. (1988) reported that significantly ($P < 0.01$) less herpes simplex virus was produced in stationary than in rotary cultures. Although the procedures of rotary cultures are more burdensome than those of stationary cultures, our data suggest that rolling of inoculated cultures should be conventionally applied in clinical virology laboratories to aid in the isolation of PCPV. In this study, there was no evidence of infection on the teats and udder in the affected cattle, which are the more common lesion sites of pseudocowpox infection. Although, the classification of parapoxviruses was formerly based on the natural host range, clinical signs, and serology (Robinson *et al*., 1992), it does not always reflect the classification revealed by molecular analysis (Inoshima *et al*., 2001) as indicated in this study.

Previously, there have been only one report describing the detection of PCPV DNA in Japan, in which PCPV DNA was detected from oral lesions of a calf in Iwate Prefecture, in the northern part of Japan, which virus isolation was unsuccessful (Yaegashi *et al*., 2013). Notably, the partial-length sequence of the *B2L* gene determined from the PCR product (accession no. AB921003) was identical to that of the present strain YG2828 (data not shown). Thus, our results confirmed that PCPV can be isolated from atypical sites besides the teats and udder, suggesting that YG2828-like PCPV may cause oral lesions in cattle. Moreover, two similar strains were isolated or detected from affected cattle in different locations, and different year, YG2828-like PCPV might be spreading among the cattle population in Japan. It is known that cattle are frequently infected with parapoxvirus subclinically (Yaegashi *et al*., 2013) and PCPV has zoonotic potential (Friederichs *et al*., 2014). Therefore, it is recommended to wear gloves for people with regular exposure to cattle mucosa. Since, PCPV is responsible for significant economic losses in the cattle production, further virological and

epidemiological studies to characterize this strain and the possibility of its spread in Japan are highly required.

5.6. LEGEND FOR FIGURES

Figure 5-1. Clinical presentation of an affected cattle with white vesicles in the mucosa under the tongue surface.

Figure 5-2. Cytopathic effect observed in BT cells after 3rd passages. The cells were observed at day 3 after inoculation. Non-infected control (a) and infected (b) cells are shown.

Figure 5-3. Alignment of the deduced amino acid sequences of the full-length *B2L* gene. Amino acids identical to the pseudocowpox virus strain YG2828 at given positions are represented by dots.

Figure 5-4. Phylogenetic tree of parapoxviruses based on the amino acid sequence of the full-length *B2L* gene (378 amino acids). The percentage bootstrap values calculated from 1000 replications are indicated above the internal nodes.

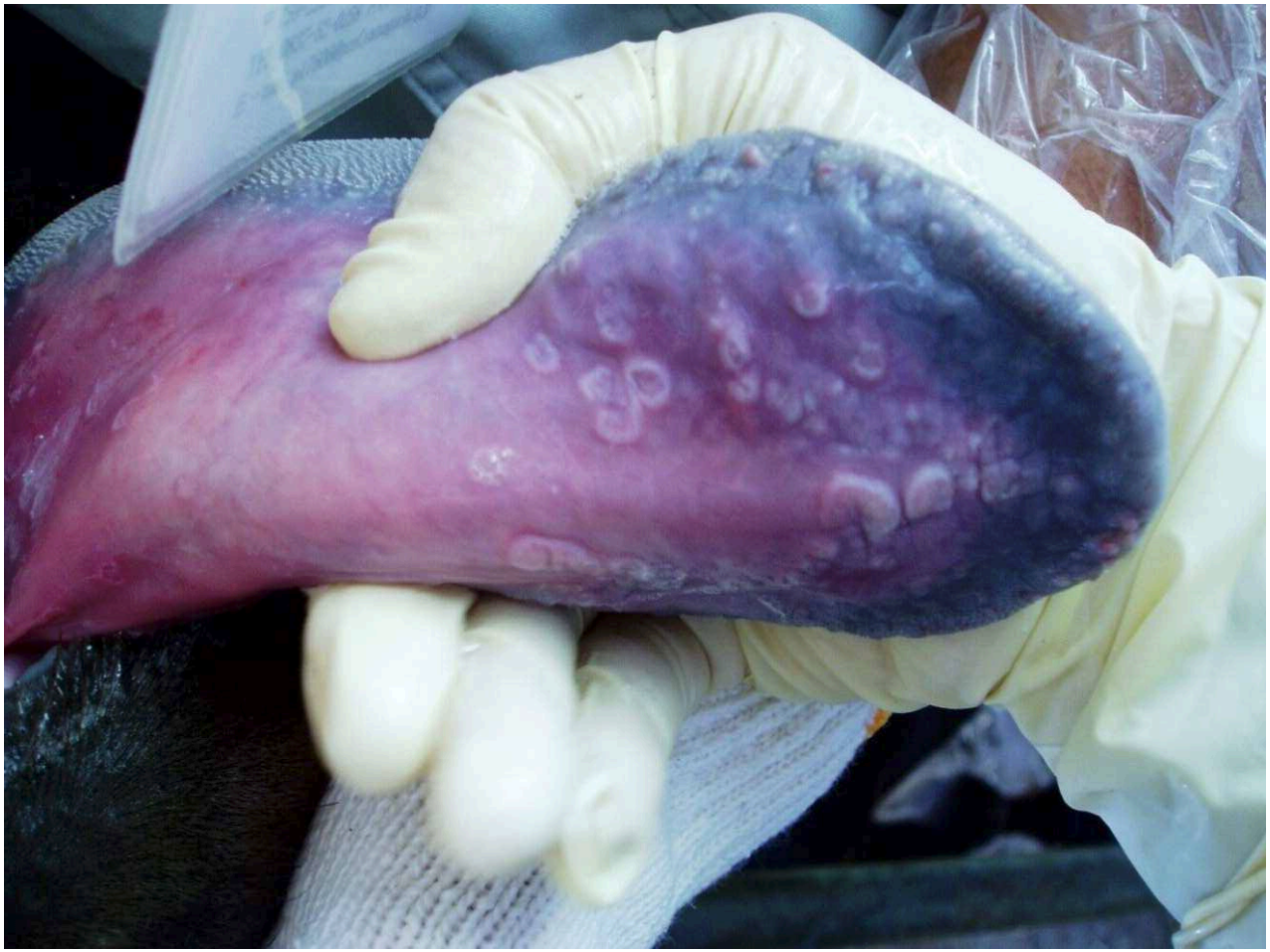

Figure 5-1. Clinical presentation of an affected cattle with white vesicles in the mucosa under the tongue surface.

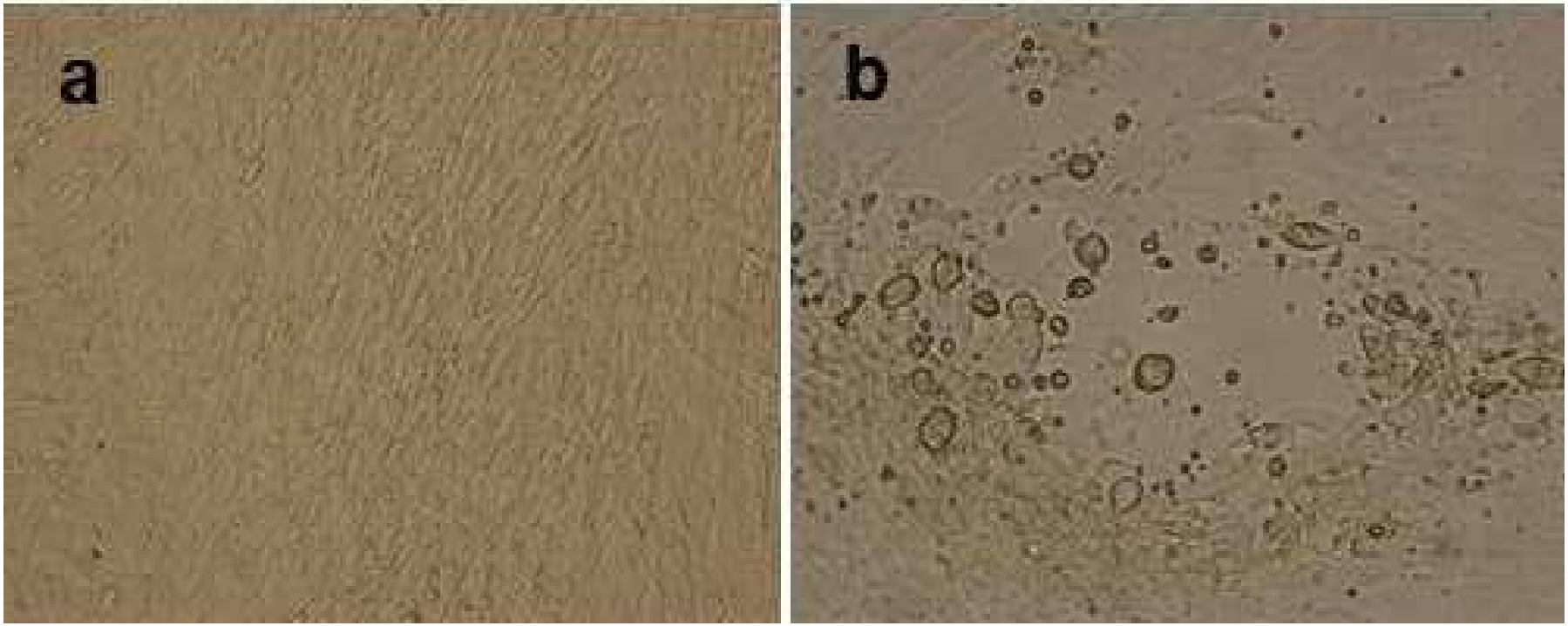

Figure 5-2. Cytopathic effect observed in BT cells at passage 3.

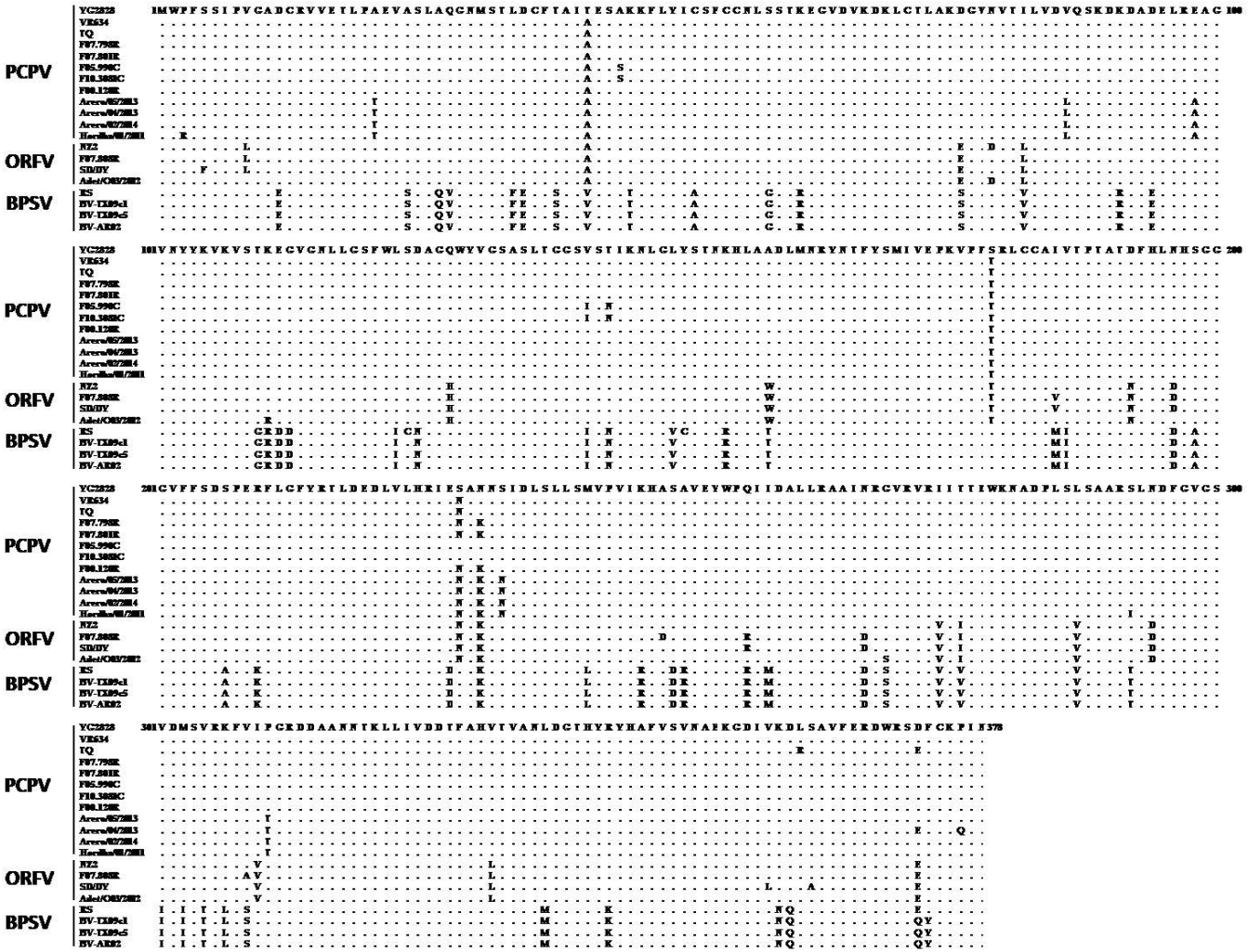

Figure 5-3. Alignment of the deduced amino acid sequences of the full-length *B2L* gene.

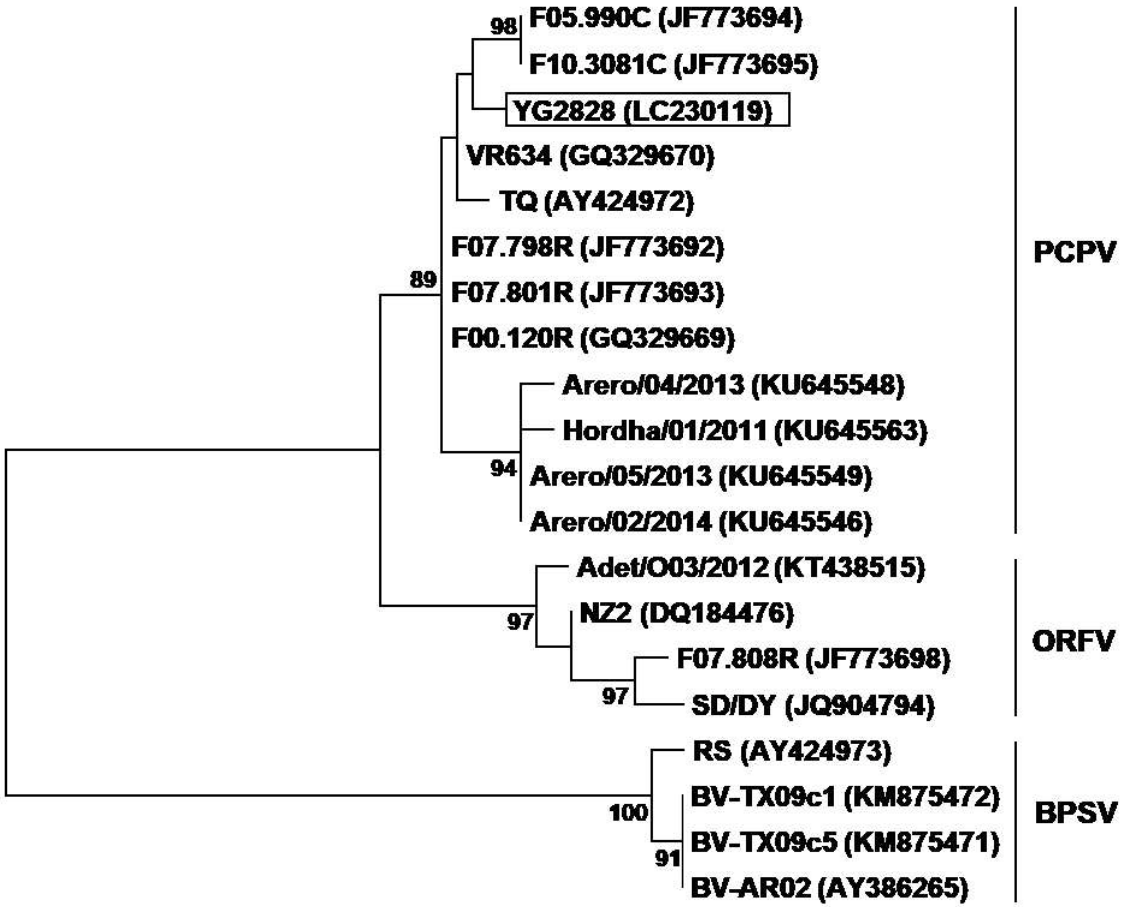

Figure 5-4. Phylogenetic tree of parapoxviruses based on the deduced amino acid sequence of the full-length *B2L* gene (378 amino acids).

Table 5-1. Nucleotide and deduced amino acid sequence identities (%) of the full-length *B2L* gene
*PCPV: pseudocowpox virus, ORFV: orf virus, BPSV: bovine papular stomatitis virus.

Virus*	Strain	Host	Nucleotide	Amino acid	Accession number
PCPV	YG2828	Cattle	—	—	LC230119
	VR634	Cattle	98.6	99.2	GQ329670
	F05.990C	Cattle	98.6	98.7	JF773694
	F10.3081C	Cattle	98.6	98.7	JF773695
	TQ	Cattle	98.2	98.7	AY424972
	F07.798R	Reindeer	98.4	98.9	JF773692
	F07.801R	Reindeer	98.4	98.9	JF773693
	F00.120R	Reindeer	98.4	98.9	GQ329669
	Arero/02/2014	Camel	97.2	97.6	KU645546
	Arero/05/2013	Camel	97.2	97.6	KU645549
	Arero/04/2013	Camel	97.0	97.1	KU645548
	Hordha/01/2011	Camel	97.0	97.1	KU645563
ORFV	NZ2	Sheep	94.1	95.0	DQ184476
	Adet/O03/2012	Sheep	94.0	95.0	KT438515
	SD/DY	Sheep	93.9	93.7	JQ904794
	F07.808R	Reindeer	93.7	93.9	JF773698
BPSV	BV-AR02	Cattle	86.1	84.7	AY386265
	BV-TX09c1	Cattle	85.8	84.7	KM875472
	BV-TX09c5	Cattle	85.8	84.7	KM875471
	RS	Cattle	85.8	84.4	AY424973

6. GENERAL CONCLUSION

Infectious diseases in cattle are caused by infections with various pathogens such as viruses, bacteria, fungi, protozoa and parasites. Although vaccine and antibiotics are administrated for the prevention and treatment, infectious diseases have not yet been well controlled. These infectious diseases cause weight loss, decrease of lactation and the death, resulting in big economic loss in farmers.

In Japan, Act on “Domestic Animal Infectious Diseases Control (Act No. 166 of 1951)” was established for prevention of diseases in livestock animals. In this law, 28 domestic animal infectious diseases and 71 notifiable infectious diseases are prescribed.

Since 1950, livestock hygiene service centers were established in each prefecture based on “Livestock Hygiene Service Centers Act (Act No. 12 of 1950)” and have contributed for prevention and diagnosis of livestock infectious diseases. In Yamaguchi Prefecture, four livestock Hygiene Service Centers (Toubu, Chubu, Seibu and Hokubu) were established, and have played important roles in diagnosis and prevention of infectious diseases in livestock in Yamaguchi.

In this thesis, infectious diseases in cattle in Yamaguchi Prefecture from 2006 to 2016 were summarized and three pathogens, Akabane virus, *C. pecorum* and pseudocowpox virus, were genetically, antigenically and histopahologically analyzed

In CHAPTER 1, infectious diseases of cattle in Yamaguchi Prefecture from 2006 to 2016 were summarized to understand the current status for prevention of the infectious diseases. In cattle in Yamaguchi Prefecture from 2006 to 2016, the number of infectious diseases with strong pathogenicity as domestic animal infectious diseases and notifiable infectious diseases were relatively small. However, the five infectious diseases, Akabane disease, bovine viral diarrhea-mucosal disease, infectious bovine rhinotracheitis, bovine leukemia and tetanus were reported and their annual incidence was higher than those in other prefectures. Also, there were large numbers of opportunistic infectious diseases. The ratio of infectious diseases was the highest in meat calves (51.7%), followed by dairy cattle (23.2%), fattening cattle (10.8%), milk calves (7.6%) and breeding cattle (6.7%). Bacteria, viruses, protozoa, fungi and parasites were detected from 72.1%, 21.9%, 2.8%, 1.9%, and 0.3% of cattle, respectively.

In CHAPTER 2, Application of Real-Time RT-PCR for Diagnosis of Akabane Disease is described. It is generally difficult to isolate Akabane virus (AKAV) and detect its gene and antigen from abnormal calves and cattle with neurological signs. Using ten clinical samples of Akabane disease obtained in Yamaguchi Prefecture from September 2011 to January 2012, the usefulness of real-time RT-PCR (rRT-PCR) in diagnosis of the disease was examined. AKAV genes were mainly detected from the brain stem, spinal cord and cerebellum of nine cattle with neurological signs and an abnormal calf by rRT-PCR. Positive correlations were found among the AKAV gene quantity, the severity of the inflammatory reaction and intensity of positive immunoreactivity in the central nervous system. Our data suggests that rRT-PCR is useful as sensitive and accurate diagnosis of Akabane disease.

In CHAPTER 3, Genetic and antigenic analysis of *Chlamydia pecorum* strains isolated from calves with diarrhea were described. *C. pecorum* (designated 22–58) was isolated in 2010 in HmLu-1 cells from the jejunum of a calf which died of necrotizing enterocolitis in Yamaguchi Prefecture, Japan. Immunohistochemical staining identified *C. pecorum* positive reactions in the jejunal villi. *C. pecorum*, designated 24–100, was isolated from the feces of a calf with diarrhea in another farm in Yamaguchi Prefecture in 2012. A significant increase in neutralizing antibody titers against *C. pecorum* was confirmed in paired sera. Nucleotide sequence identities of *omp1* genes of the 2 isolates were 100%. The isolates were genetically and antigenically more closely related to *C. pecorum* Bo/Yokohama strain isolated from cattle with enteritis in Japan than to the other prototype strains, Bo/Maeda isolated from cattle with pneumonia and Ov/IPA isolated from sheep with polyarthritis. These results indicate that *C. pecorum* strains similar to 22–58 and 24–100 might be endemic in Yamaguchi Prefecture and cause enteric disease in cattle.

In CHAPTER 4, First isolation and genetic characterization of pseudocowpox virus from cattle in Japan were described. Pseudocowpox virus (PCPV) infects cattle worldwide with zoonotic potential but has not been isolated in Japan. Thus, the epidemiological status of PCPV infection in cattle is undetermined. In May 2016, cattle in a farm in Yamaguchi Prefecture showed white vesicles and hyperemia in the mucosa under the tongue surface, but not on the teats and coronary cushions. A parapoxvirus was isolated from the oral lesion swab and was genetically characterized based on the

full-length sequence of *B2L* gene encoding viral envelope. Phylogenetic analysis showed that the isolated virus was classified into PCPV. This case indicates its potential spread in Japan. This is the first report of isolation of PCPV in Japan.

7. ACKNOWLEDGMENTS

The present studies were carried at Yamaguchi Chubu Livestock Hygiene Service Center, Japan, from 2004 to 2014, and 2016 to 2018.

First of all, the author would like to show his greatest appreciation to his supervisor, **Dr. Ken Maeda** (Laboratory of Veterinary Microbiology, Joint Faculty of Veterinary Medicine, Yamaguchi University) for providing him this precious opportunity to study as a Ph.D. with the invaluable support and advice. The author is very grateful to all of his co-supervisors, **Dr. Hiroyuki Iwata** (Laboratory of Veterinary Hygiene, Joint Faculty of Veterinary Medicine, Yamaguchi University) , **Dr. Makoto Yamakawa** (National Institute of Animal Health), **Dr. Hiroshi Shimoda** (Laboratory of Veterinary Microbiology, Joint Faculty of Veterinary Medicine, Yamaguchi University), and **Dr. Masako Andoh** (Laboratory of Veterinary Public Health, Joint Faculty of Veterinary Medicine, Kagoshima University) for giving him insightful comments, suggestions and useful discussion on his study. The author is very thankful to all of his higher-ups, **Gentaro Takeya, Shinji Matsuda, Akihiro Kawado, Shoji Sakata, Masakazu Miyoshi, Yutaka Ishikawa, Sachiko Kuniyoshi, Fuminori Yanagisawa and Yoshio Ogawa** for giving him encourages and critical advices on his experiment. The author wants to thank to his office members for their support in his experiments and making him stay comfortable and joyful during his days in office.

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10. PUBLICATION LIST

10.1. Original Article

Ohtani A, Kubo M, Shimoda H, Ohya K, Iribe T, Ohishi D, Endoh D, Omatsu T, Mizutani T, Fukushi H, Maeda K (2015) Genetic and antigenic analysis of *Chlamydia pecorum* strains isolated from calves with diarrhea. *J Vet Med Sci*; **77**(7):777-782

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10.2. Brief Report

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10.3. Review

大谷研文, 前田　健 (2016) *Chlamydia pecorum* 感染による子牛の下痢症. 家畜診療 **64**:209-215

学位論文要旨
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Studies on the infectious diseases of cattle in Yamaguchi
山口県における牛の感染症に関する研究

牛の感染症は様々な病原体の感染によって引き起こされ、下痢や呼吸器症状による体重減少、泌乳量低下、死亡等を引き起こすため、農場に大きな経済的損失を与える。農場では消毒、ワクチンを使用した予防等、様々な感染症対策の取り組みがなされているが、感染症の発生を十分に抑えられていない現状にある。

本研究は牛感染症の診断技術向上や疫学調査実施を目的とし全4章から構成される。

第1章　2006年から2016年の山口県における牛の感染症発生状況

2006年から2016年までに山口県内の牛で発生した感染症について、山口県中部家畜保健衛生所に感染症の原因究明を依頼され、何らかの病原体の関与が確認された 462 症例を対象に、発生状況、病原体の検出状況等をまとめた。監視伝染病のような病原性が強い感染症の発生は比較的少ないが、発生した5種類の届出伝染病、アカバネ病(14頭) 、牛ウイルス性下痢・粘膜病(17 頭)、牛伝染性鼻気管炎(11 頭)、牛白血病(176 頭)、破傷風(2 頭)については、発生した年の年間発生率が他の都道府県と比べて高い傾向にあった。全体的には、日和見感染症の発生が非常に多いことが判明した。用途別では子牛 59.3% (肉用子牛51.7%、乳用子牛7.6%)、乳用牛23.2%、肥育牛10.8%、繁殖牛6.7%の順に発生が多かった。症状別では呼吸器症状 27.6%、死亡 19.8%、下痢 17.1%、乳房炎 12.6%の順に多く、これらが全体の77%を占めた。病原体は細菌72.1%、ウイルス21.9%、原虫2.8%、真菌1.9%、寄生虫0.3%の順に多く検出され、細菌とウイルスが全体の94%を占めた。

以下の章では、第1章で確認されたアカバネウイルス、*Chlamydia pecorum*及び偽牛痘ウイルスについて詳細に解析した。

第2章　アカバネ病診断におけるリアルタイムRT-PCRの応用

アカバネ病の診断において、体形異常を示す子牛や神経症状を示す牛からのウイルス分離、ウイルス遺伝子検出及び抗原検出が困難なことが多い。2011年9月から2012年1月に山口県において本病が10例発生し、これらの検体を用いてリアルタイムRT-PCRの有用性を検討した。神経症状を呈した牛や死産子牛の中枢神経系(CNS)からアカバネウイルス(AKAV)遺伝子を検出できた。特にウイルス分離が困難で、初乳未摂取子牛の血清中から抗体を検出する以外に診断法がない体形異常死産子牛からAKAV遺伝子を検出できたことの意義は大きい。CNSのAKAV遺伝子量と非化膿性脳脊髄炎の程度及び免疫組織化学染色(IHC)の強度、非化膿性脳脊髄炎の程度とIHCの強度間に正の相関がみられた。CNSを材料としたリアルタイムRT-PCRは従来の検査法より感度が高く、得られた成績はアカバネ病の診断精度の向上に貢献することが期待される。

第3章　下痢を呈した子牛から分離された*Chlamydia pecorum*の遺伝子解析と抗原解析

2010年、山口県の1農場において下痢を呈し死亡した子牛の空腸から*C. pecorum* (22-58株)が分離され、抗*C. pecorum*家兎血清を用いたIHCによって空腸絨毛に陽性反応が認められた。2012年には、別の農場で下痢を呈した子牛の糞便から*C. pecorum* (24-100株)が分離され、ペア血清において分離株に対する中和抗体の有意上昇が確認された。山口県において下痢を呈した牛からの*C. pecorum*分離症例の報告はなく、2株について遺伝子解析と抗原解析を実施した。*ompA*遺伝子の部分配列の解析から、2株の塩基配列は一致し、国内で肺炎

の牛から分離された株や米国で多発性関節炎のめん羊から分離された株とは相同性が低く、国内で腸炎の牛から分離されたBo/Yokohama株と最も相同性が高かった。イムノブロット解析により22-58株は抗Bo/Yokohama株家兎血清と強く反応した。22-58株と24-100株の分離年は異なり、疫学的関連も確認されていない。本章ではBo/Yokohama株に類似した*C. pecorum*株が山口県において流行し、牛に腸疾患を引き起こすことが確認された。

第4章　日本の牛から初めての偽牛痘ウイルスの分離とその遺伝的特性

偽牛痘ウイルス(PCPV)は偽牛痘を引き起こし、多くの国で報告されているが、日本の牛におけるPCPV感染の疫学状況は明らかにされていない。2016年5月、山口県内の1農場の牛の舌表面粘膜下に白色水疱と充血がみられた。乳頭や蹄冠に異状は認められなかった。口腔内病変部の拭い液から回転培養法によってPCPVが分離された。分離ウイルスは*B2L*遺伝子の全長配列の解析から、米国やフィンランドの牛から分離されたPCPVに遺伝的に近縁であった。今後、分離ウイルスの特徴や日本における拡がりについて、さらなるウイルス学的、疫学的研究が必要と考えられる。本症例は、日本でPCPVが分離された最初の報告である。

以上の「アカバネ病診断精度の向上」、「*C. pecorum* の牛下痢症への関与の証明」、「国内における初めての牛からの PCPV の分離」により、牛感染症の理解が深まり牛感染症対策への応用展開が期待される。