表7　各株のマウスに対する病原性（腹腔内接種）

株	マウス系統	接種菌数 (cfu)	接種個体数	症状発現接種後日数	
				体重減少	斃死
BH8859	C57BL/6JJmsSlc	58	3	-	-
N9	C57BL/6JJmsSlc	190	3	-	-
N1915	C57BL/6JJmsSlc	154	3	-	-
C.M.V.103	C57BL/6JJmsSlc	252	3	-	-
N335-64	C57BL/6JJmsSlc	2	3	-	-
	C57BL/6JJmsSlc	143	3	-	-
Kf water#23	C57BL/6JJmsSlc	48	3	-	-
Kf71	C57BL/6JJmsSlc	542	3	-	-
Chiba	C57BL/6JJmsSlc	298	3	4	4
Ebina	C57BL/6JJmsSlc	686	3	4	5
GIEM-Miura	C57BL/6JJmsSlc	3	3	-	-
	C57BL/6JJmsSlc	357	3	-	-
Kato	C57BL/6JJmsSlc	4780	3	1	3-4
KU-1	C57BL/6JJmsSlc	367	3	3	4
Nikaido	C57BL/6JJmsSlc	100000	2	2	3-4
NVF1	C57BL/6JJmsSlc	328	3	3	4-5

体重減少：接種時の体重より5%減少した場合とした。

-：症状認められず

表8　皮内接種によるマウスの症状の推移

株	接種個体数	接種菌(cfu)	接種後日数 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	個体数 体重減少	個体数 斃死	MTD
NVF1	8	3.8					▽	▽▽▽	▽▽		▽●●●	●●●●				▽	●	8	8	9.3
	8	38						▽▽▽▽▽	▽	▽▽●●●●	●●●●							8	8	7.5
	8	324					▽▽▽▽▽▽	▽	▽●●●●●	●●	●							8	8	6.5
	8	3240					▽▽▽▽▽▽▽	▽	●●	●●●●●	●							8	8	6.9
KU-1	8	26						▽	▽▽▽▽▽▽	●●●●	●●	●						7	7	7.6
	8	260	▽					▽▽	▽▽▽	▽▽●●●●●●	●●							8	8	7.3
Kato	8	30					▽▽	▽▽▽▽▽▽	●	●●●●●●●								8	8	6.9
	8	300					▽▽▽	▽▽▽▽	▽	●●●●●●●●								8	8	7.0
Sami	8	166					▽	▽▽▽▽▽▽	▽●	●●●	●●●●							8	8	7.4
	8	1660				▽	▽▽▽	▽▽▽▽	●●	●●●●●●								8	8	6.8
Yama	8	174						▽	▽	▽▽▽▽▽		▽	●●●		△△	●	△	8	4	10.8
	8	1740					▽▽	▽▽	▽▽▽		●			●	●●●△△	●		7	6	11.3
Ebina	8	200					▽▽			▽▽	▽▽	▽	●△△△△	△△				7	1	−
	8	2000							▽	▽▽▽	▽△	▽	△△△	△△				6	0	−
Chiba	8	68					▽	△										1	0	−
	8	680																0	0	−
Nikaido	3	200				▽	▽▽						△△					3	0	−

●：斃死、▽：体重減少、△：体重回復（接種日の体重より5%低下した場合と体重減少とし、その回復を体重回復とした）

MTD: Mean Time to Death

肉眼観察

陽性　　弱陽性　　陰性

顕微鏡下像

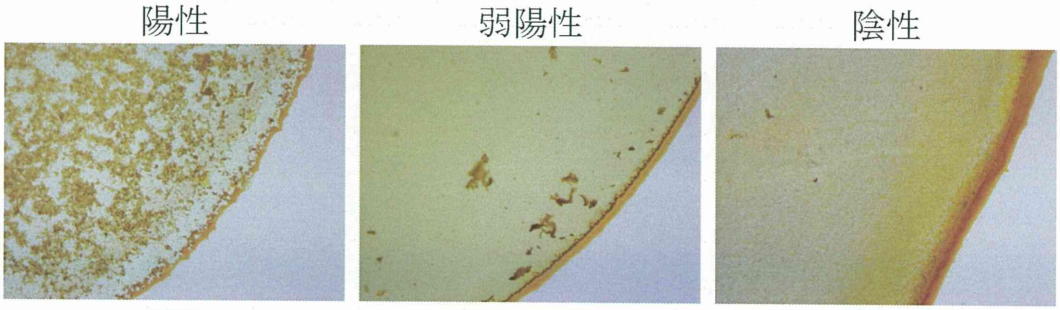


図1　アクリフラビン反応（一部のみ）

C.M.V.103、LVS、N19、N503、RV、TungliaoおよびJap株の菌液がアクリフラビン溶液と混合後、1分以内に凝集像を呈したため陽性と判定した。SchuおよびOotakeは混合3分後に凝集像を呈したため弱陽性とした。他の株は混合5分後まで凝集が認められなかった。顕微鏡下像の倍率40倍で撮影した。

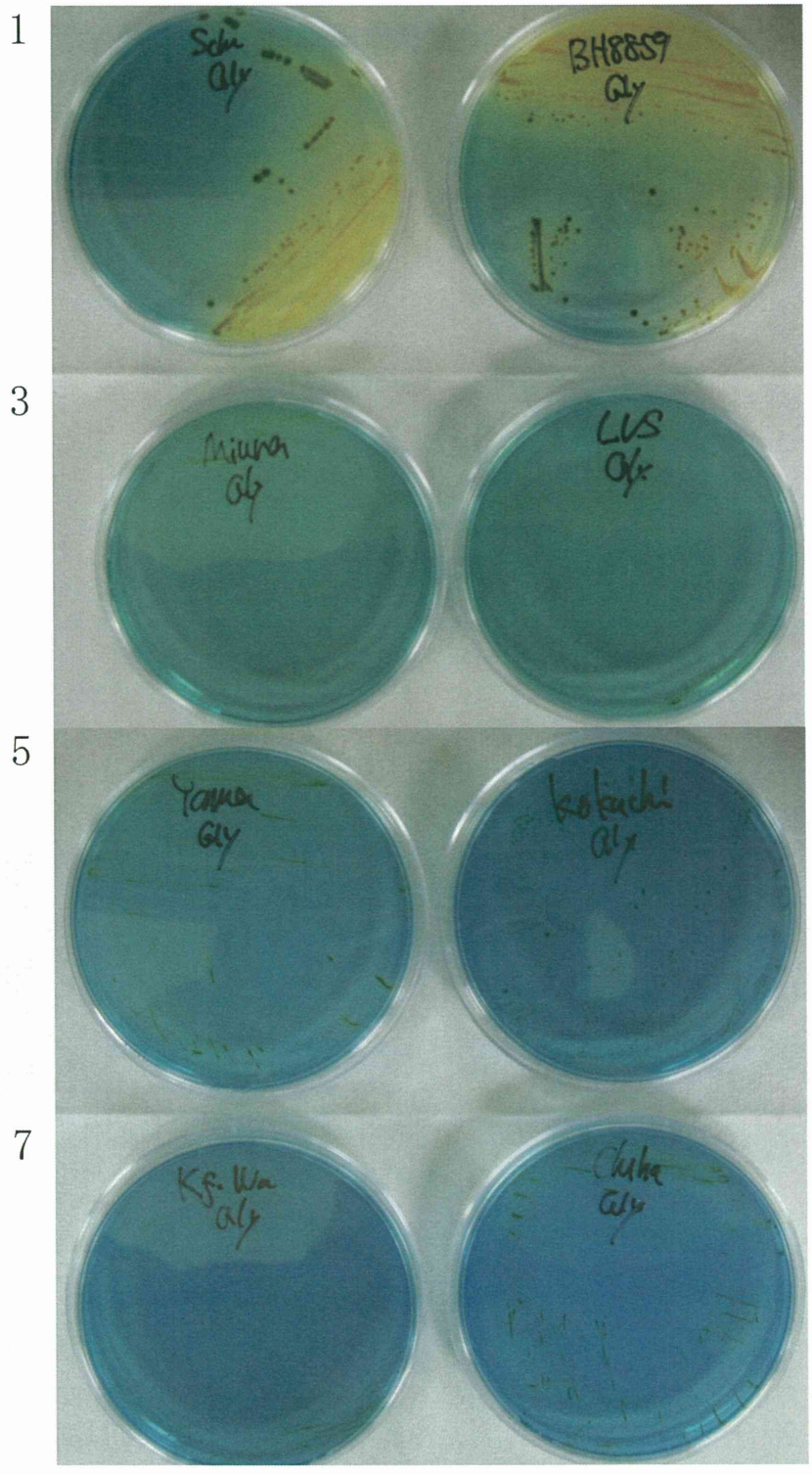


図2　各株のグリセロール発酵能（一部のみ）

1: Schu, 2: BH8859, 3:GIEM-Miura, 4:LVS, 5:Yama, 6:Kokuchi, 7: Kf water#23, Chiba

Schu、BH8859および38（データ示さず）はグルコースを含まないグリセロール添加培地に良好に発育し、培地を青色から黄色に変色させた。他の株はコロニーは認めるも発育が悪く、培地の変色は認められなかった。

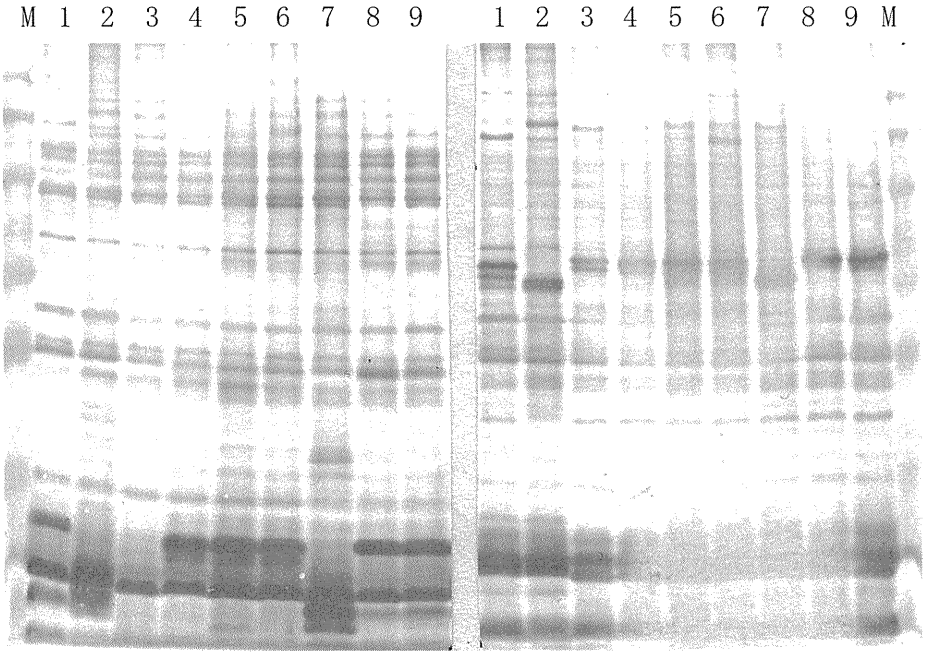
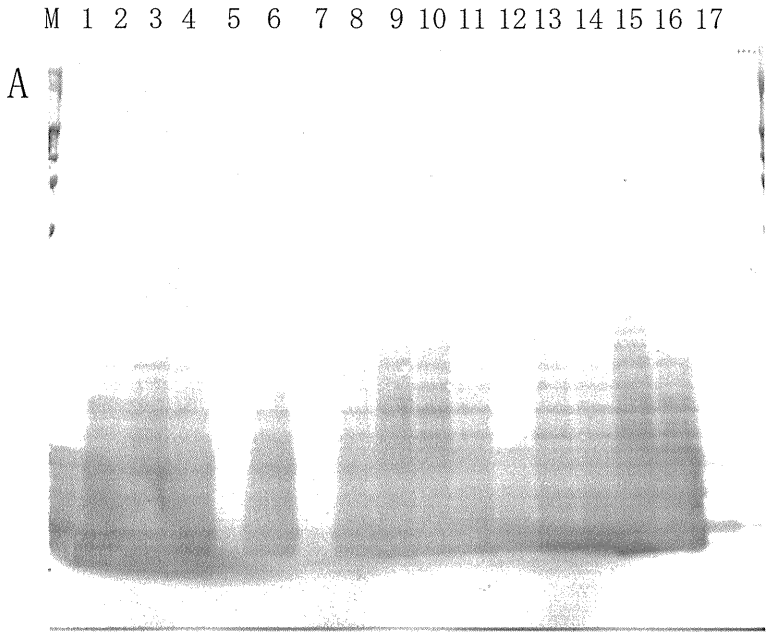
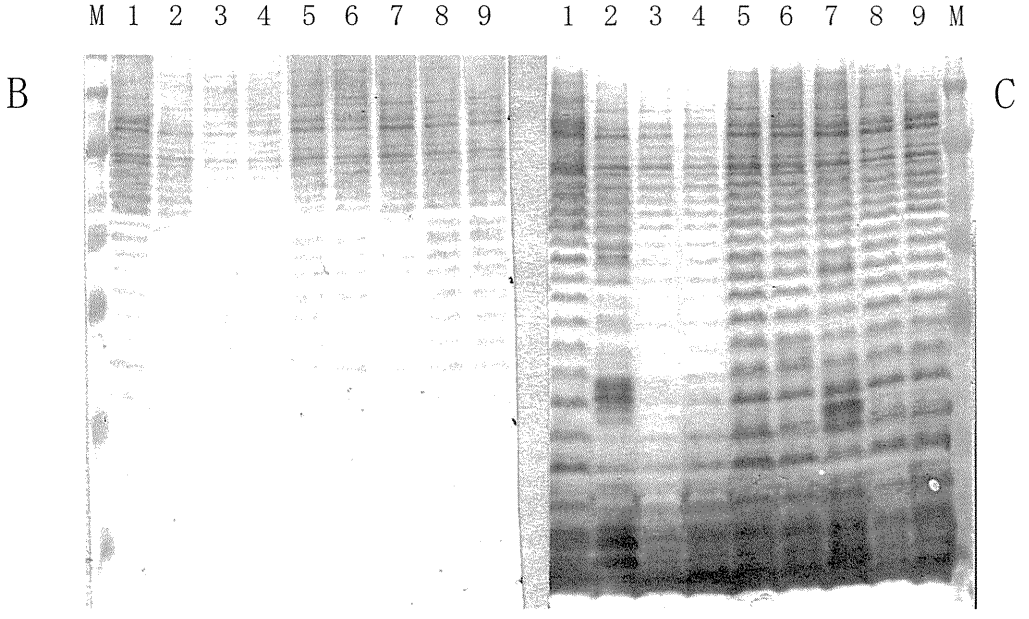


図3　供試株の免疫血清との反応

反応血清 A: Yama株免疫マウス血清、B: *F. novicida* U112株免疫マウス血清
抗原　lane 1:Schu, 2:BH8859, 3:Kf water#23, 4:N335-64, 5:N9, 6:N1915, 7:Ebina, 8:Yama, 9:NVF1, M:Marker WIDE-VIEW™ Prestained Protein Size Maraker(Wako)



lane 1: LVS、2: 38、3: Schu、4: N9、5: N503、6: N1915、7: Tungliao、8: Azumaya、9: Ebina、10: Kokuchi、11: GIEM-Miura、12: Ootake、13: Sami、14: Yama、15: Yato96、16: Yato107、17: U112(*Francisella novicida*)



lane 1:Schu, 2:BH8859, 3:Kf water#23, 4:N335-64, 5:N9, 6:N1915, 7:Ebina, 8:Yama, 9:NVF1,M:Marker WIDE-VIEW™ Prestained Protein Size Maraker(Wako)

図4　供試株のLPS認識モノクローナル抗体との反応

反応抗体　AおよびC：FB11B、B：M14B11

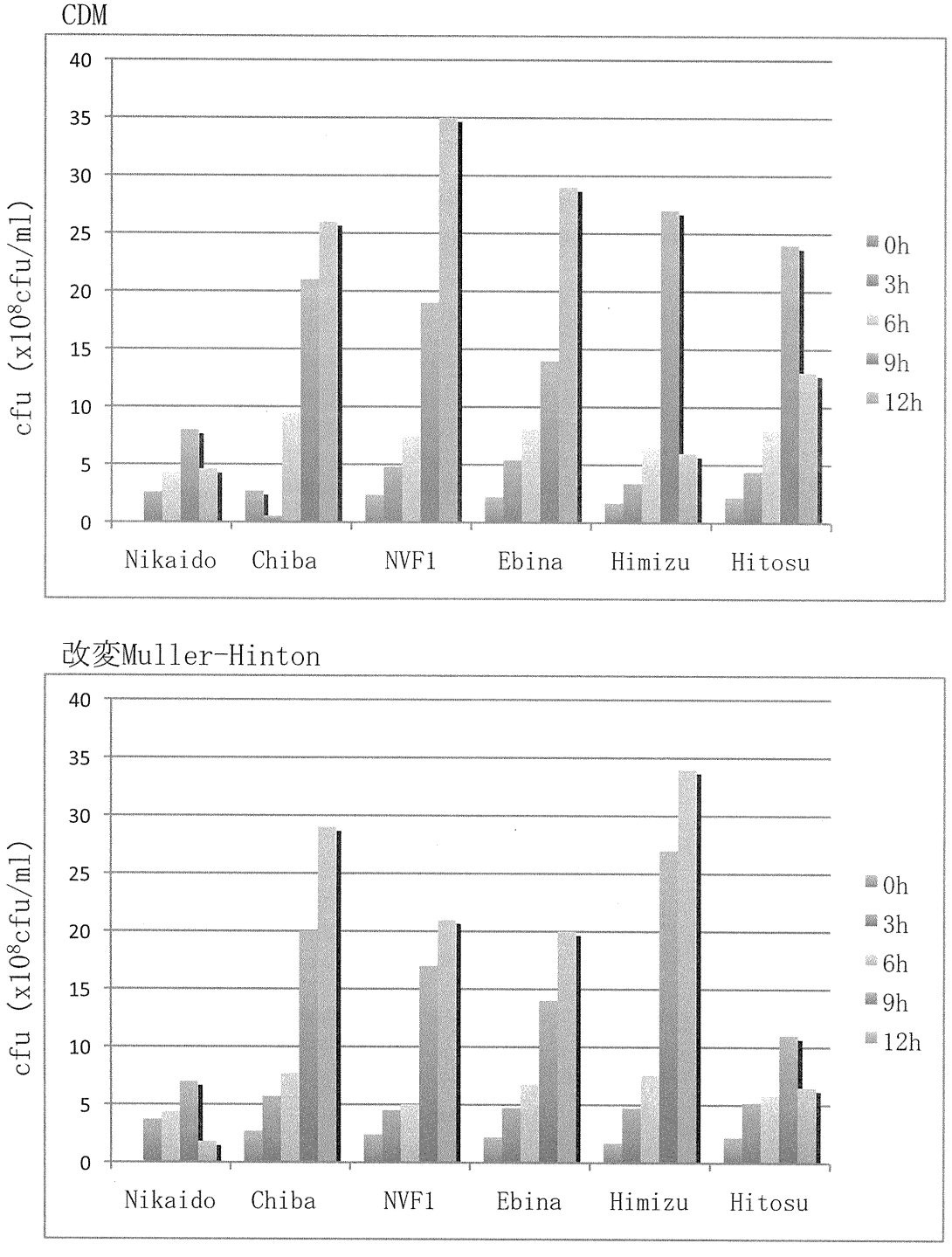


図5　培地各種における供試株のcfuの変化

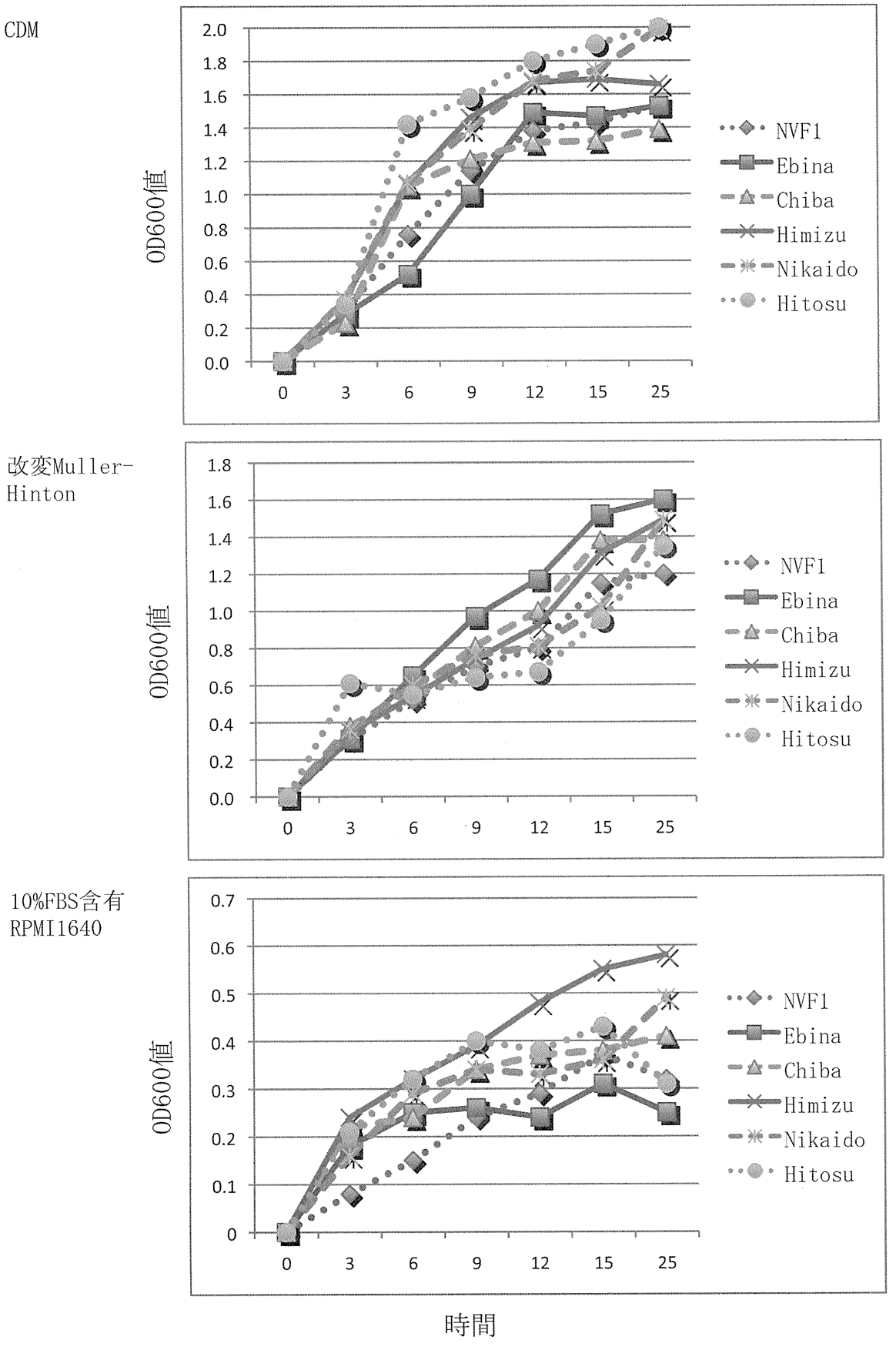


図6　培地各種におけるOD600値の経時的変化

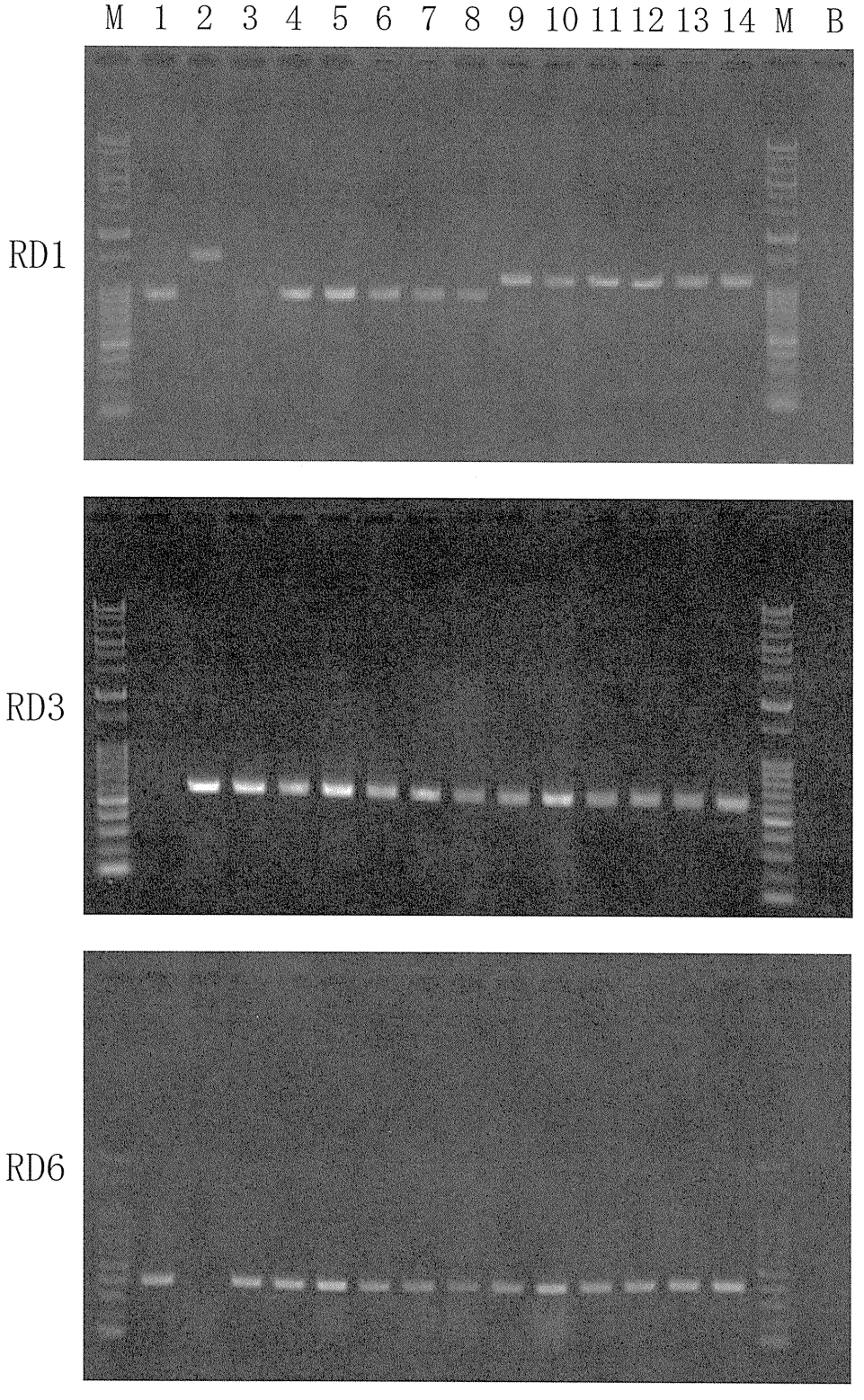


図7　RD1、3および6のPCR産物の電気泳動像

lane 1:38, 2:BH8859, 3:Kf water#23, 4:Kf 71, 5:N335-64, 6:N9, 7:N1915, 8:LVS, 9:Chiba, 10:Kato, 11:GIEM-Miura, 12:KU-1, 13:NVF1, 14:Yama, M:Marker, Gene Ladder Wide 2(Nippon Gene), B: blank

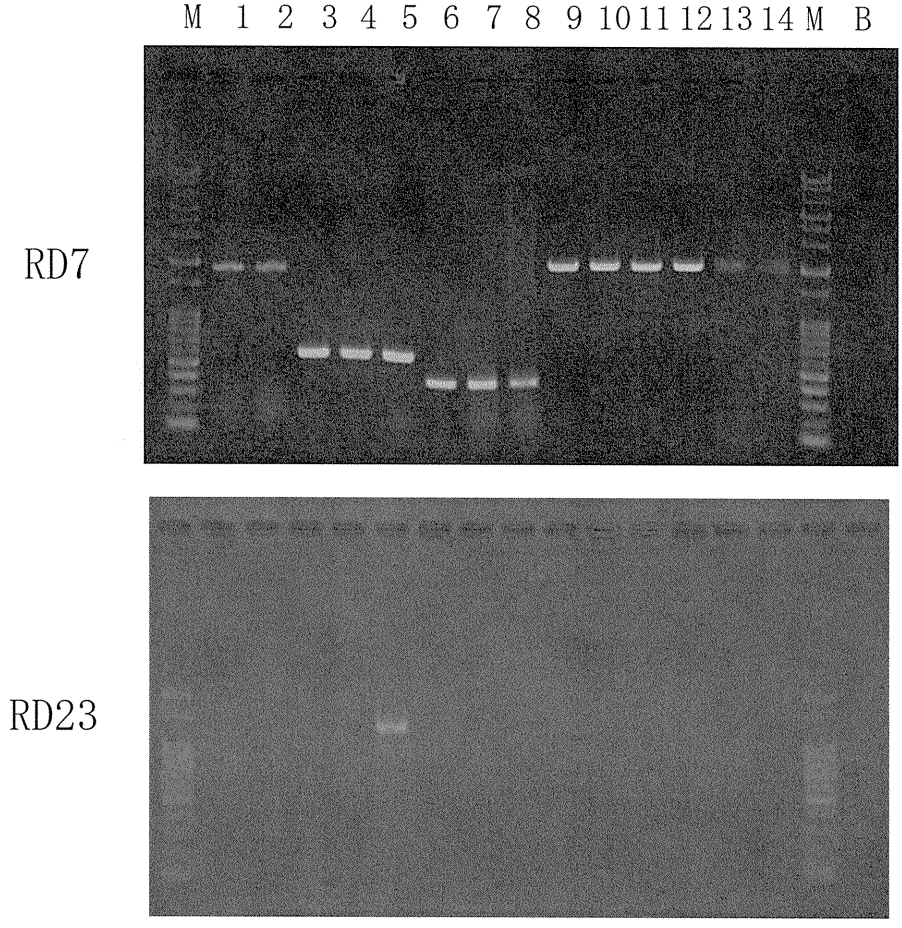


図8　RD7および23のPCR産物の電気泳動像

lane 1:38, 2:BH8859, 3:Kf water#23, 4:Kf 71, 5:N335-64, 6:N9, 7:N1915, 8:LVS, 9:Chiba, 10:Kato, 11:GIEM-Miura, 12:KU-1, 13:NVF1, 14:Yama, M:Marker,Gene Ladder Wide 2(Nippon Gene), B: blank

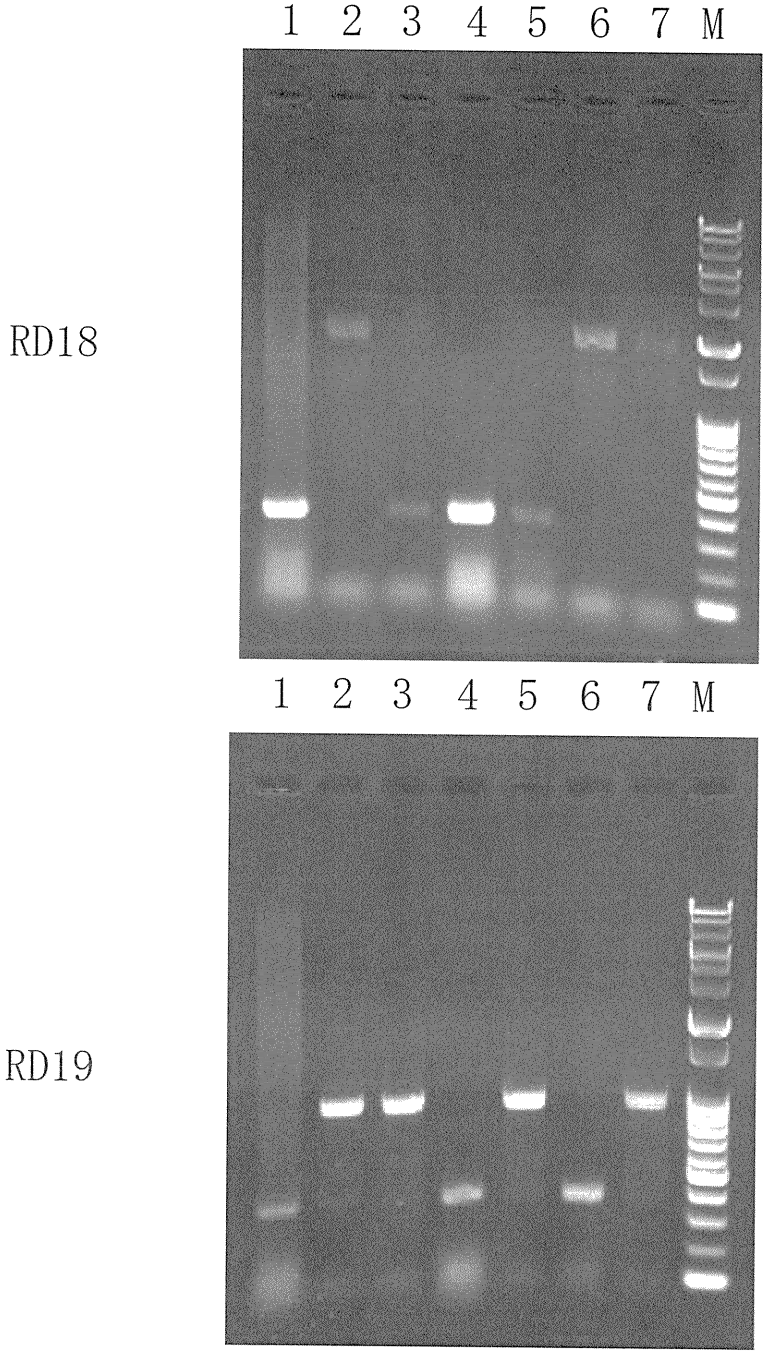


図9　RD18および19のPCR産物の電気泳動像

lane 1:LVS, 2:N19, 3:N503, 4:C.M.V.103, 5:Tungliao, 6:Jap, 7: Schu, M:Gene Ladder Wide 2(Nippon Gene)

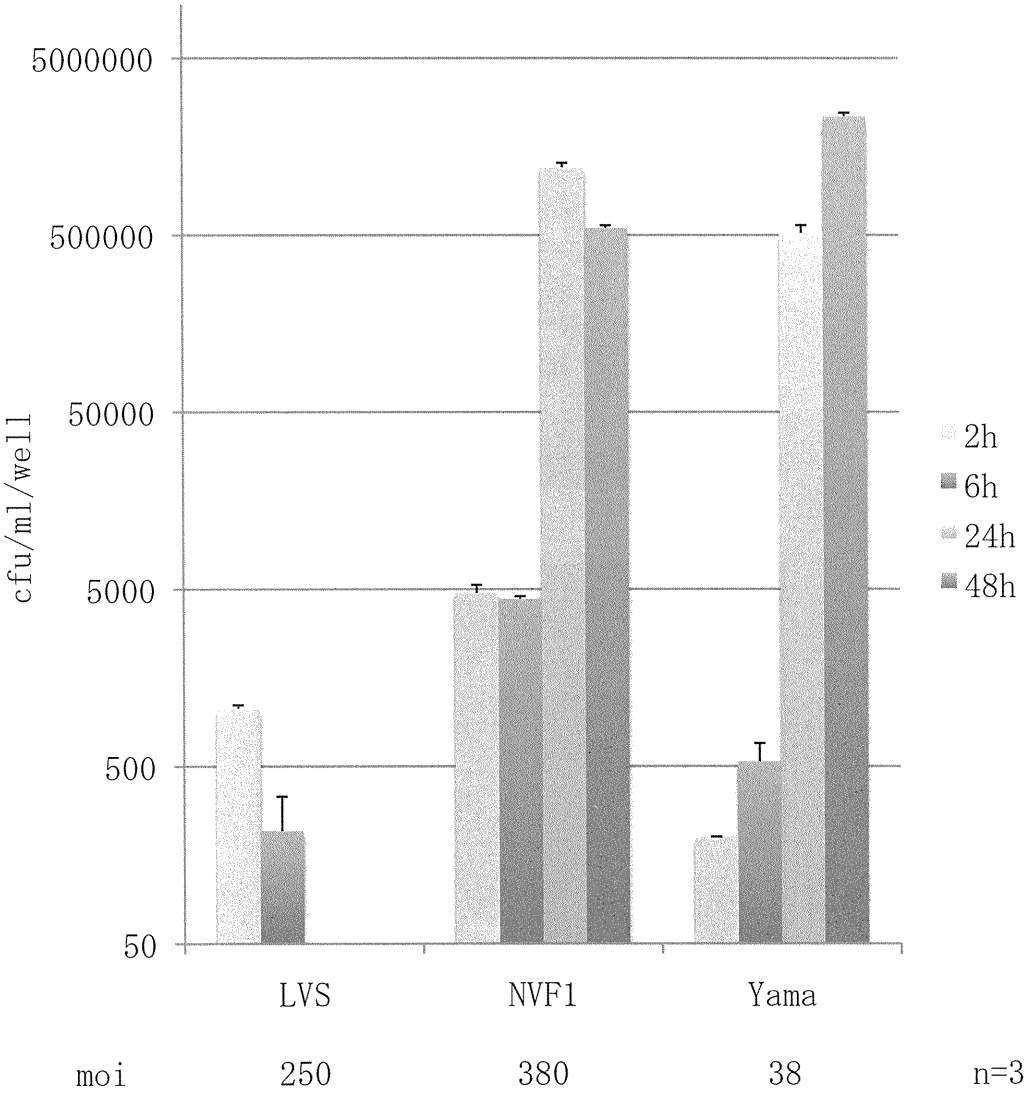


図10　J774.1細胞内における病原株と弱毒株の増殖性

病原性が確認されているYama、新鮮分離株のNVF1、およびマウスに非病原性であったLVSの細胞内菌数を経時的に測定した。

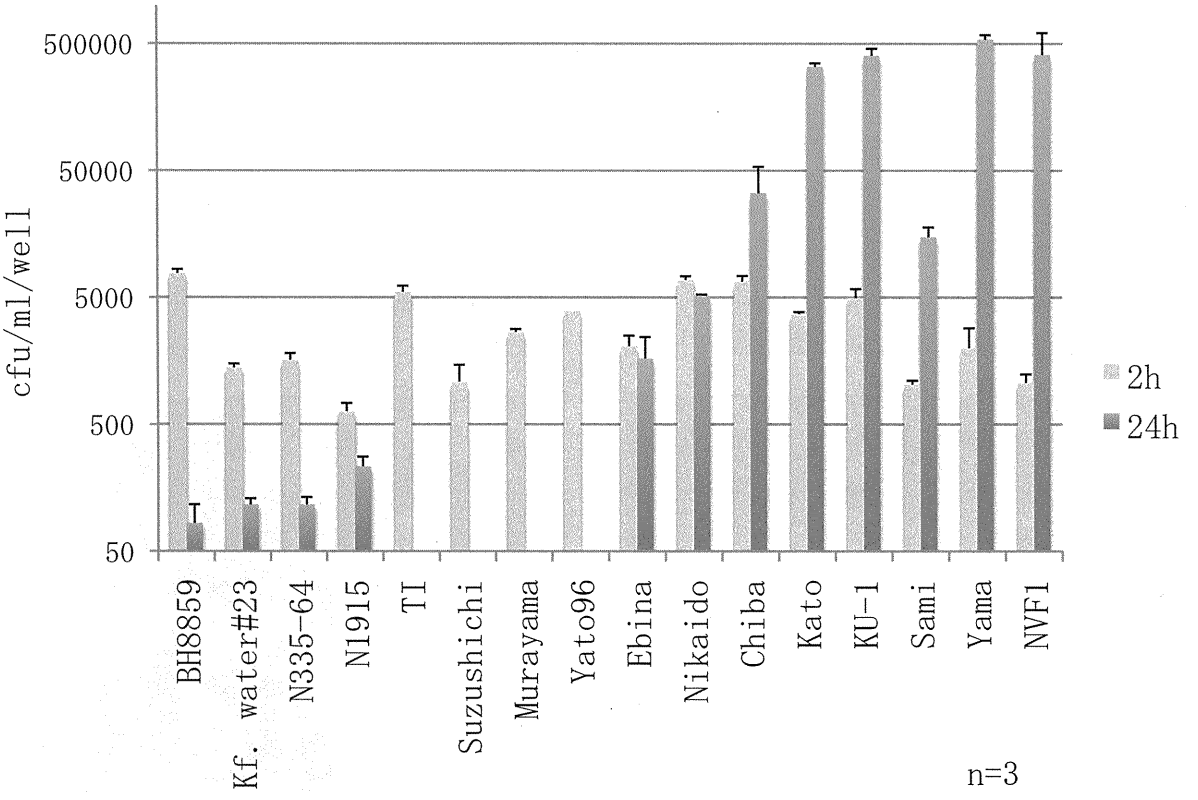


図11　J774.1細胞内における各株の増殖性（一部のみ）

各株ともmoiを10^2cfu に調整した。接種後2時間のcfuが50以下であった株については遠心吸着にて強制的に感染させた。

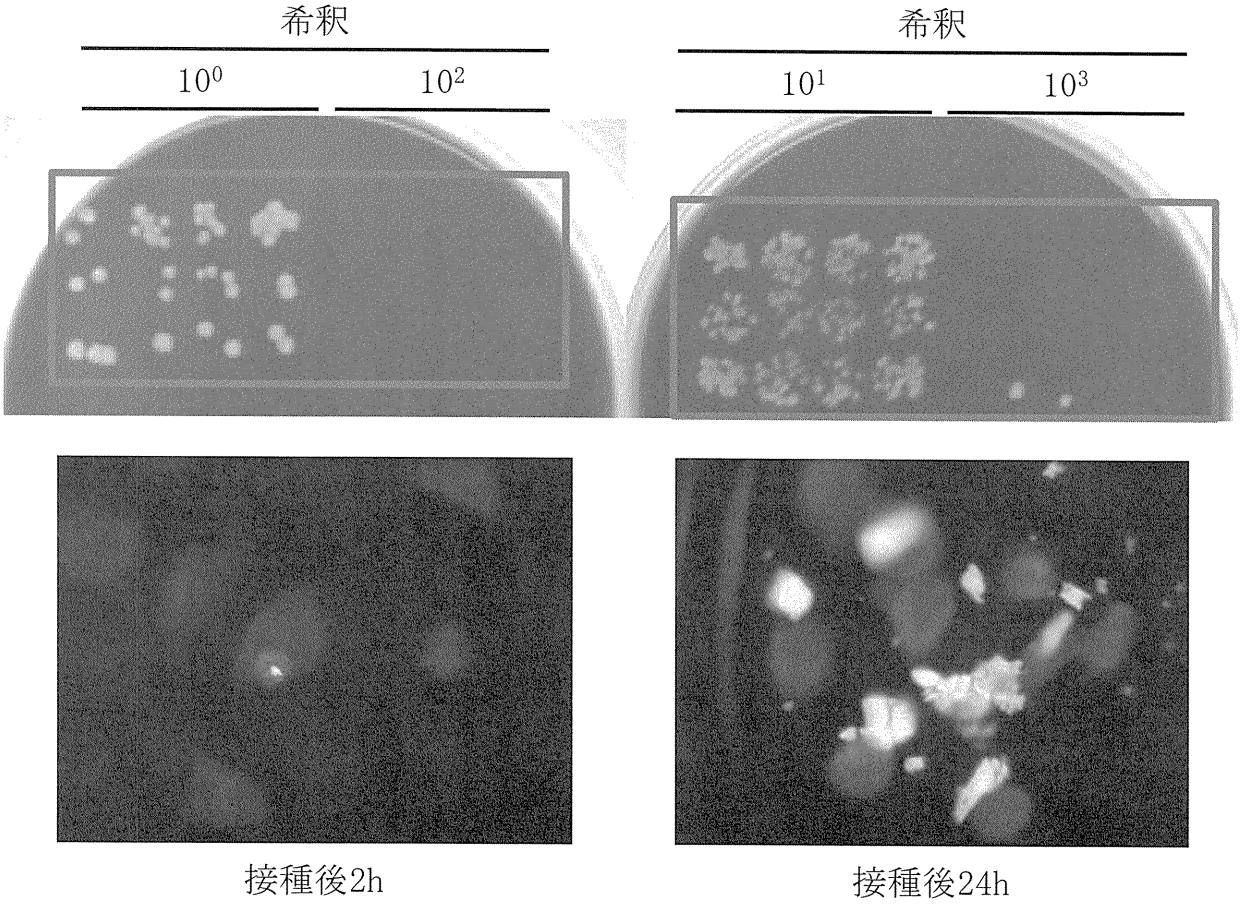


図12　細胞内高増殖性株のcfu増加および蛍光顕微鏡観察像

cfuは10倍段階希釈液を5μl x 4spotずつチョコレート(II)寒天に滴下、培養し、3日目に測定した。蛍光顕微鏡観察はFITC標識免疫ウサギ血清を用いた。

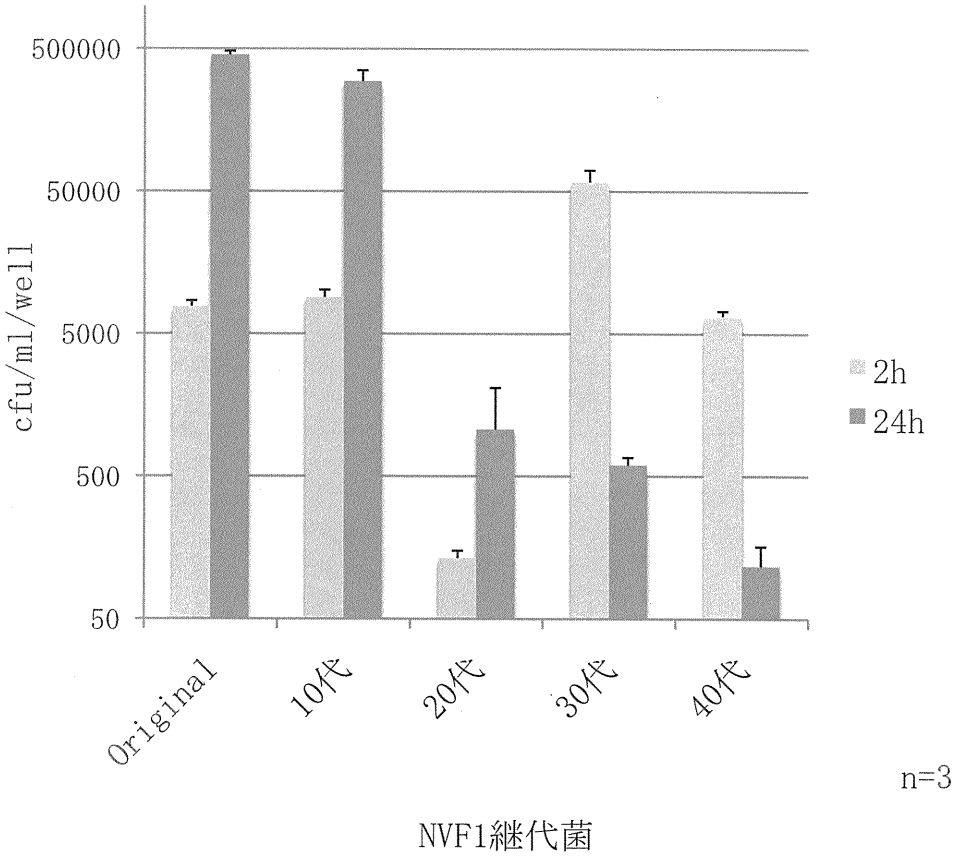


図13　NVF1人工培地継代菌のJ774.1細胞内増殖性

斃死ノウサギの脾臓よりEugonチョコレート寒天培地で分離したNVF1をOriginalとして、Eugonチョコレート寒天培地で2-3日おきに継代した。

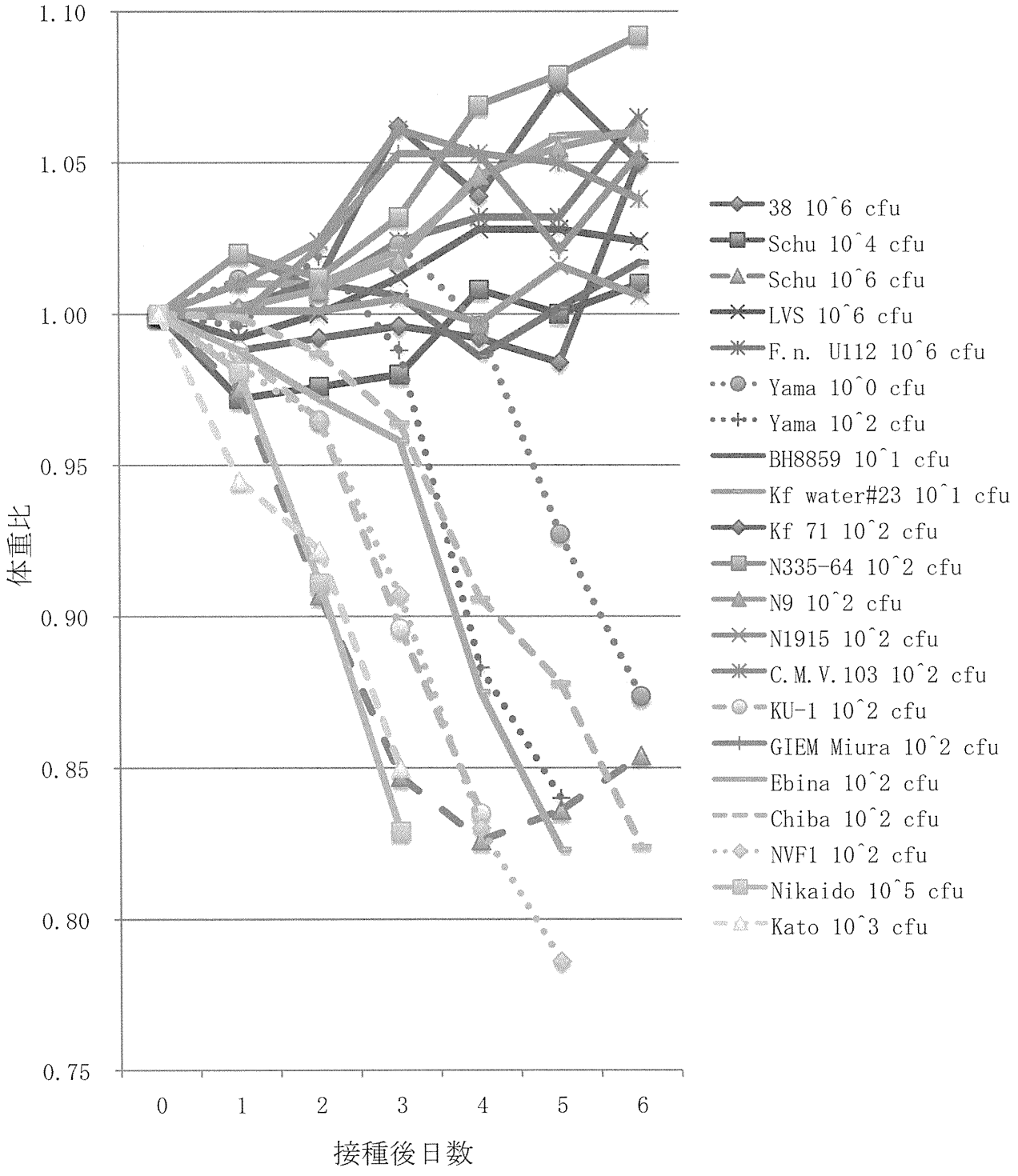


図14　*F. tularensis* 接種マウスの体重推移（i.p.）

接種日の体重を1として接種後の体重増減比を示した。38、LVS、Schu、*F. novicida* U112およびYamaはBALB/cマウス、BH8859、Kf water#23、Kf 71、N335-64、N9、N1915、C.M.V.103、KU-1、GIEM Miura、Ebina、Chiba、NVF1、NikaidoおよびKatoはC57BL/6JJに接種した。凡例は株名および接種菌量を示す。SchuおよびNikaido接種マウスは2匹、他の株接種マウスは各群3匹のマウスを用いた。

Ⅱ．研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
Akitoyo Hotta, Kiyoshi Tanabayashi, Yoshie Yamamoto, Osamu Fujita, Akihiko Uda, Toshio Mizoguchi, Akio Yamada	Seroprevalence of tularemia in wild bears and hares in Japan	Zoonoses and Public Health	59(2)	89-95	2012

Ⅲ. 研究成果の刊行物・別刷

ORIGINAL ARTICLE

Seroprevalence of Tularemia in Wild Bears and Hares in Japan

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Impacts

- Serological assays for tularemia were performed with 431 Japanese black bears and 293 Japanese hares samples.
- All eight seropositive samples were originated from Japanese black bears from the Tohoku district, northeastern region of the Honshu, Japan.
- Japanese black bears can be used as a sentinel for tularemia.

Keywords:
Francisella tularensis; tularemia; wild animals; seroprevalence

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Summary

Tularemia is a zoonotic disease caused by *Francisella tularensis*. The distribution of the pathogen in Japan has not been studied well. In this study, seroprevalence of tularemia among wild black bears and hares in Japan was determined. Blood samples collected from 431 Japanese black bears (*Ursus thibetanus japonicus*) and 293 Japanese hares (*Lepus brachurus*) between 1998 and 2009 were examined for antibodies against *F. tularensis* by micro-agglutination test (MA) or enzyme-linked immunosorbent assay. By subsequent confirmatory tests using western blot (WB) and indirect immunofluorescence assay (IFA), eight sera from Japanese black bears were definitely shown to be seropositive. All of these eight bears were residents of the northeastern part of main-island of Japan, where human tularemia had been reported. On the other hand, no seropositive Japanese hares were found. These results suggest that Japanese black bears can serve as sentinel for tularemia surveillance and may help understand the distribution of *F. tularensis* throughout the country. This is the first report on detection of antibody to *F. tularensis* in black bears of Japan.

Introduction

Tularemia is a zoonotic disease caused by *Francisella tularensis*, highly infective, intracellular gram-negative coccobacilli. It is primarily a disease of wild animals: mainly lagomorphs and rodents. The disease occurs throughout the northern hemisphere including North America, Russia, Europe and Japan. In North America and Europe, 100–200 human tularemia cases are reported every year (Ellis et al., 2002). Humans are infected through contact with infected animals, arthropod bites, ingestion of contaminated water or food, and inhalation of infective aerosols (Ellis et al., 2002). The clinical type and severity of the disease is dependent on the route of infection. Predominant symptoms are high fever, enlarged lymph nodes, and ulcer at the site of bacterial entry (Ellis et al., 2002). In animals, the severity of the disease varies among species. In susceptible animals such as mice severe collapses are followed by a fatal septicaemia. Other animal species such as cats, dogs and cattle are relatively resistant to the infection (Hopla, 1974).

Understanding of the distribution of the pathogen in animal populations is of particular importance when studying zoonoses. The seroprevalence of *F. tularensis* in wild animals in North America and Europe has been reported for bears (Binninger et al., 1980; Chomel et al., 1998), hares (Mörner et al., 1988; Frölich et al., 2003), rabbits (Shoemaker et al., 1997; Berrada et al., 2006) and wild boars (Al Dahouk et al., 2005). These data are indispensable to assess the risk of future occurrence of tularemia in humans and domestic animals as well as to identify the natural reservoir of *F. tularensis*.

In Japan, tularemia was first reported in 1924, and approximately 1400 human cases have been reported since then (Ohara et al., 1991). The annual incidence of tularemia has decreased from the middle of the 1960s and it became extremely rare thereafter (Ohara et al., 1996).