参考文献

1) Akao, N., Tsukidate, S., Mori, Y., Kajiyama, T., Kondo, K. and Fujita, K. The lethal effect of freezing on spirurid nematode larvae in firefly squids, *Watasenia scintillans*, 寄生虫誌, 44, 321-324, 1995.
2) Alvarez-Guerrero, C. and Alba-Hurtado, F. Effect of some physical factors on the viability of third-stage *Gnathostoma binucleatum* larvae, J. Food Prot., 74, 844-848, 2011.
3) 姉尾秀貫・北川政次郎・岩本正一 冷凍鱒肉中裂頭条虫の幼虫は生活力ありや, 水産講習所試験報, 20, 123-128, 1925.
4) Bier, J. W. Experimental anisakiasis: cultivation and temperature tolerance determination, J. Milk Food Technol., 39, 132-137, 1976.
5) Eguchi, S. Studies on *Dibothriocephalus latus*, with special reference to its life history in Japan, *Proceedings of the Fifth Pacific Science Congress*, 5, 4145-4149, 1929.
6) Fan, P. C. Viability of metacercariae of *Clonorchis sinensis* in frozen of salted freshwater fish, Int. J. Parasitol., 28, 603-605, 1998.
7) Fayer, R. and Nerad, T. Effects of low temperatures on viability of *Cryptosporidium parvum* oocysts, Appl. Environ. Microbiol., 62, 1431-1433, 1996.
8) 深沢 平・輿水 馨・金井恒夫・原田泰治・中村常仁 斎藤和雄・菅谷重雄・徳富剛二郎 食肉中におけるトキソプラズマ虫体の温度に対する抵抗性試験, 日獣医会誌, 17, 25-27, 1964.
9) Gustafson, P. V. The effect of freezing on encysted *Anisakis*, J. Parasitol., 39, 585-585, 1953.
10) Hilwig, R. W., Cramer, J. D. and Forsyth, K. S. Freezing times and temperatures required to kill cysticerci of *Taenia saginata* in beef, Vet. Parasitol., 4, 215-219, 1978.
11) 鎌田洋一 ザルコシスティスが含まれる馬肉による食中毒, 日食微, 29, 47-52, 2012.
12) 川田茂宏 *Anisakis*症に関する研究-*Anisakis*幼虫の抵抗性について-, 大阪医大誌, 26, 224-244, 1968.
13) 小林昭夫・鈴木了司・安田一郎・熊田三由・石井俊雄・小川初枝・小宮義孝 低温に対する鉤虫卵の抵抗性に関する研究, 寄生虫誌, 8, 637-641, 1959.
14) Kotula, A. W., Dubey, J. P., Sharar, A. K., Andrews, C. D., Shen, S. K. and Lindsay, D. S. Effect of freezing on infectivity of *Toxoplasma gondii* tissue cysts in pork, J. Food Prot., 54, 687-690, 1991.
15) Kotula, A. W., Sharar, A. K., Paroczay, E., Gamble, H. R., Murrell, K. D. and Douglass, L. Infectivity of *Trichinella spiralis* from frozen pork, J. Food Prot., 53, 571-573, 1990.
16) 桑原直徳 五種トリコモナスの培養ならびに抵抗力について, 朝鮮医学誌, 29, 1227-1253, 1931.
17) Lacour, S. A., Heckmann, A., Macé, P., Grasset-Chevillot, A., Zanella, G., Vallée, I., Kapel, C. M. and Boireau, P. Freeze-tolerance of *Trichinella* muscle larvae in experimentally infected wild boars, Vet. Parasitol., in press, 2013.
18) Mahbubani, M. H., Bej, A. K., Perlin, M., Schaefer, F. W. Ⅲ, Jakubowski, W. and Atlas, R. M. Detection of *Giardia* cysts by using the polymerase chain reaction and distinguishing live from dead cysts, Appl. Environ. Microbiol., 57, 3456-3461, 1991.
19) 村田以和夫・宮沢貞雄・国 守利・中嶋陽一・渋谷智晃・中西弘 北海道のスケトウダラ *Teragra chalcograma*, 千葉県産マサバ *Pneumatophorus Japonicus*由来*Anisakis* Ⅰ型幼虫の保存温度, 薬味, 調味料および香辛料に対する抵抗性, 東京都衛研報, 38, 13-21, 1987.
20) Salminen, K. The effect of high and low temperature treatments on the infectiveness of *Diphyllobothrium latum* with regard to public health. Acta. Vet. Sc., 32(Suppl.), 1-29, 1970.
21) Sotelo, J., Rosas, N. and Palencia, G. Freezing of infested pork muscle kills cysticerci, J. Am. Med. Assoc., 256, 893-894, 1986.
22) Sterling, C. R. and Ortega, Y. R. *Cyclospra*: an enigma worth unraveling, Emerg. Inf. Dis., 5, 48-53, 1999.
23) 杉山 広・柴田勝優・森嶋康之・山崎 浩・川上 泰 肺吸虫の感染を予防するためのサワガニ冷凍条件の検討, Clin. Parasitol., 23, 57-59, 2012.
24) Taira, K., Saitoh, Y., Okada, N., Sugiyama, H. and Kapel, C. M. O. Tolerance to low temperatures of *Toxocara cati* larvae in chicken muscle tissue, Vet. Parasitol., 26, 383-386, 2012.
25) Theodoropoulos, G., Kapel, C. M., Webster, P., Saravanos, L., Zaki, J. and Koutsotolis, K. Infectivity, predilection sites, and freeze tolerance of *Trichinella* spp. in experimentally infected sheep, Parasitol. Res., 86, 401-405, 2000.
26) 津田守道 肺吸虫 *Paragonimus westermani*の生物学的研究 (2) 肺吸虫被嚢幼虫の抵抗について, 寄生虫誌, 8, 164-173, 1959.
27) 山田源二 *Anisakis*症の感染予防に関する研究, 大阪医大誌, 20, 1-29, 1971.
28) 山浦 常 トキソプラズマ・オーシストに関する研究, Ⅰ, 低温および乾燥に対する抵抗性について, 寄生虫誌, 25, 80-86, 1976.
29) 山崎 繁・富田俊雄・山下六郎 蛔虫卵の低温に対する抵抗力に関する研究, 寄生虫誌, 3, 240-244, 1954.
30) 温泉川肇彦 生食用生鮮食品を共通食とする病因物質不明有症事例の解明をめざして, 日食微, 29, 43-46, 2012.

D.E. 考察および結論

1．食品衛生に係わる各種法令の国際比較

食品媒介寄生虫に対する食品衛生上の対応が必要な現状では，他の法規に委ねるのではなく，「食品衛生法」において，寄生虫に係る食品の規格基準を定める必要があると考えられた．

2. 寄生原虫・寄生蠕虫の冷凍耐性に関する調査・研究

2-1. 寄生原虫・寄生蠕虫を殺滅する冷凍条件についての文献調査

ICMSF がリストアップしながらも，検討が進んでいない寄生虫（赤痢アメーバと蟯虫）に関しては，冷凍による殺滅効果を検討し，飲食に伴う健康被害が防止されるかを，今後明らかにする必要がある．

一方で，低温耐性が非常に強い寄生虫の存在が知られる．ある種の旋毛虫がその例となる．この種は我が国の野生動物にも寄生しており，野生動物から飼育豚に汚染が広がる危険性もある．感染源となり得る豚肉の汚染を「と畜場法」に則して検査する必要があり，また旋毛虫類に係る食肉（豚肉）の規格基準の導入を我が国でも急ぐ必要がある．

2-2. 寄生虫の殺滅に有効な冷凍条件に関する研究

宮崎肺吸虫のメタセルカリアは，ウェステルマン肺吸虫よりも冷凍耐性が高く，中間宿主サワガニを-18℃で 100 分間冷凍しても，実験マウスへの感染を完全には予防できなかった．有効な冷凍条件を再検討する必要がある．また猫回虫以外の *Toxocara* 属回虫に対する冷凍条件の検討が必要である．

3. 寄生虫による食品汚染に関する調査研究

3-1. マサバおよびゴマサバにおけるアニサキス同胞種の寄生状況

3-2. キンメダイにおけるアニサキスの寄生状況

3-3. サバ等の加工食品からのアニサキスの検出状況

深海魚のキンメダイからも，As や Ap とは異なる稀有な人体寄生種 *A. physeteris*ga が多数検出された．注意が必要である．サバの加工食品等からもアニサキス As が検出された．死滅虫体であるために，感染の危険性はないが，アニサキス・アレルギーなどの健康被害の原因にもなり得ることから，対策が必要と考えられた．

F. 健康危険情報

平成 23 年 4 月 26 日に「サワガニ体内に寄生する肺吸虫」について，厚生労働省健康危機管理調整官宛に通知した．

G. 研究発表

１．論文発表

1. Sugiyama, H., Shibata. K., Morishima. Y., Muto, M., Yamasaki, H., Kawakami, Y. Current status of lung fluke metacercarial infection in freshwater crabs in the Kawane area of Shizuoka Prefecture, Japan. Journal of Veterinary Medical Science 75, in press, 2013.
2. 杉山　広．生食による寄生虫感染症のリスク，In *生食のおいしさとリスク*（一色賢司　編）．エヌ・ティ・エス，印刷中，東京，2013.
3. 杉山　広．食品による寄生動物感染症 7. 蠕虫感染症 (2) 肺吸虫．日本防菌防黴学会誌，41，印刷中，2013.
4. Sugiyama, H., Singh, T.S. and Rangsiruji, A. *Paragonimus* (Chapter. 39). In *Molecular Detection of Human Parasitic Pathogens*, (Liu, D.-Y., ed.), pp. 421-433, CRC press, Boca Raton, 2012.
5. Taira, K., Saitoh, Y., Okada, N., Sugiyama, H., Kappel, C.M.O. Tolerance to low temperatures of *Toxocara cati* larvae in chicken muscle tissue. Veterinary Parasitology 189, 383-386, 2012

6. 杉山 広, 柴田勝優, 森嶋康之, 山﨑浩, 川上 泰. 肺吸虫の感染を予防するためのサワガニ冷凍条件の検討. Clinical Parasitology 23, 57-59, 2012.
7. 杉山 広, 森嶋康之, 山﨑 浩, 春日文子. 食用として販売されていたサワガニからの肺吸虫メタセルカリアの検出（続報）. 病原微生物検出情報 32, 172-173, 2011.
8. 鈴木 淳, 村田理恵, 貞升健志, 甲斐明美. アニサキス感染事例およびサバ加工食品におけるアニサキスの寄生状況, Clinical Parasitology 22, 82-84, 2011.
9. 杉山 広, 森嶋康之, 山﨑 浩, 柴田勝優, 川上 泰. 肺吸虫の感染を予防するためのサワガニ加熱条件の検討. Clinical Parasitology 21, 43-45, 2010.
10. 杉山 広. 食品と寄生虫感染症. 日本食品衛生学雑誌 51, 285-291, 2010.
11. 杉山 広. 食品媒介寄生虫による食中毒. 日本食品微生物学雑誌 27, 1-7, 2010.

２．学会発表

1. Sugiyama H. Current status of paragonimiasis in Japan: Infections associated with exotic dietary habits. The 7th Seminar on Food- and Water-borne Parasitic Zoonoses, December 15, 2012, Bangkok, Thailand.
2. 杉山 広, 柴田勝優, 森嶋康之, 山﨑浩, 川上 泰. 肺吸虫の感染を予防するためのサワガニ冷凍条件の検討. 第23回日本臨床寄生虫学会, 東京, 2012年6月.
3. 杉山 広. アニサキスを原因とする食中毒. 第153回日本獣医学会学術集会・公衆衛生学分科会シンポジウム, 大宮, 2012年3月.
4. Taira K, Saitoh Y, Kapel C.M.O. *Toxocara cati* larvae persist and remain high infectivity in muscles of experimentally infected chickens. The 23rd International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP), Buenos Aires, August 2011.
5. Sugiyama, H., Umehara, A., Kawakami, Y., Ohmae, H., Ooi, H.-K., Uchida, A. Molecular identification of *Anisakis* type I larvae from hairtail fish in Taiwan and Japan. The 23rd International Conference of the World Association for Advancement of Veterinary Parasitology (WAAVP 2011), Buenos Aires, August 2011.
6. 鈴木 淳, 村田理恵, 貞升健志, 甲斐明美. アニサキス感染事例およびサバ加工食品におけるアニサキスの寄生状況, 第22回日本臨床寄生虫学会, 東京, 2011年7月.
7. 杉山 広, 森嶋康之, 山﨑 浩, 柴田勝優, 川上 泰. 肺吸虫の感染を予防するためのサワガニ加熱条件の検討. 第21回日本臨床寄生虫学会, 栃木, 2010年6月.

H. 知的財産権の出願・登録状況

特許および実用新案登録共になし.

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版
Hiroshi Asakura, Yoshika Momose, and Fumiko Kasuga	Enterohemorrhagic Escherichia coli – Its Control from a Viewpoint of Food Safety –	Journal of Disaster Research	Vol.6No.4	426-434	2011
Hiroshi Asakura, Keiko Kawamoto, Yumiko Okada, Fumiko Kasuga, Sou-ichi Makino, Shigeki Yamamoto, Shizunobu Igimi	Intrahost passage alters SigB-dependent acid resistance and host cell-associated kinetics of Listeria monocytogenes	Infection, Genetics and Evolution	12	94-101	2012
H.Asakura, E.Saito, Y.Momose, T.Ekawa, M.Sawada, A.Yamamoto, A.Hasegawa, J.Iwahori, T.Tsutsui, K.Osaka, T.Matsushita, M.Kakinuma, K.Motoyama, Y.Hayama, H.Kitamoto, S.Igimi and F.Kasuga	Prevalence and growth kinetics of Shiga toxin-producing Escherichia coli (STEC) in bovine offal products in Japan	Epidemiol. Infect.	140	655-664	2012
Hiroshi Asakura, Tomoya Ekawa, Naoki Sugimoto, Yoshika Momose, Keiko Kawamoto, Sou-ichi Makino, Shizunobu Igimi, Shigeki Yamamoto	Membrane topology of Salmonella invasion protein SipB confers osmotolerance	Biochemical and Biophysical Research Communications	426	654-658	2012
Kusumoto A, Asakura H, Kawamoto	General stress sigma factor RpoS influences time required to enter the viable but non-culturable state in Salmonella enterica.	Microbiol Immunol.	*In press*		2012
Asakura H, Ekawa T, Sugimoto N, Momose Y, Kawamoto K, Makino S, Igimi S, Yamamoto S	Providencia alcalifaciens causes barrier dysfunction and apoptosis in tissue cell culture: potent role of lipopolysaccharides on diarrheagenicity.	Food Addit Contam.	*In press*		2013
杉山　広	食品と寄生虫感染症	日本食品衛生学雑誌	Vol.51,No.6	285-291	2010
杉山　広	食品媒介寄生虫による食中毒	日本食品微生物学雑誌	27(1)	1-7	2010
杉山 広,森嶋康之, 山﨑　浩, 柴田勝優, 川上　泰	肺吸虫の感染を予防するためのサワガニ加熱条件の検討	Clinical Parasitology	Vol.21,No.1	43-45	2010
杉山　広, 森嶋康之, 山﨑　浩, 春日文子	食用として販売されていたサワガニからの肺吸虫メタセルカリアの検出(続報)	病原微生物検出情報	Vol.32,No.6	172-173	2011
鈴木　淳, 村田理恵, 貞升健志, 甲斐明美	アニサキス感染事例およびサバ加工食品におけるアニサキスの寄生状況	Clinical Parasitology	Vol.22,No.1	82-84	2011
Kensuke Taira, Yasuhide Saitoh, Natsuki Okada, Hiromu Sugiyama, Christian M.O. Kapel	Tolerance to low temperatures of Toxocara cati larvae in chicken muscle tissue	Veterinary Parasitology	189	383-386	2012

杉山 広, 柴田勝優, 森嶋康之, 山﨑浩, 川上 泰	肺吸虫の感染を予防するためのサワガニ冷凍条件の検討	Clinical Parasitology	Vol.23,No.1	57-59	2012
Hiromu SUGIYAMA, Katsumasa SHIBATA, Yasuyuki MORISHIMA, Maki MUTO, Hiroshi YAMASAKI and Yasushi KAWAKAMI	Current Status of Lung Fluke Metacercarial Infection in Freshwater Crabs in the Kawane Area of Shizuoka Prefecture, Japan	Journal of Veterinary Medical Science	75		2013
杉山 広	食品による寄生動物感染症 7. 蠕虫感染症 (2) 肺吸虫	日本防菌防黴学会誌	Vol.41,No.3	165-171	2013

書籍

著者氏名	タイトル	書籍名	出版社	ページ	出版
(Chapter. 39を執筆) Hiromu Sugiyama, Takhellambam Shantikumar Singh, and Achariya Rangsiruji	Paragonimus(Chapter. 39)	Molecular Detection of Human Parasitic Pathogens, (Liu, D.-Y., ed.)	CRC press, Boca Raton	421-433	2012
杉山 広	生食による寄生虫感染症のリスク	生食のおいしさとリスク(一色賢司編)	エヌ・ティ・エス,東京	*印刷中*	2013

III. 研究成果の刊行物・別刷

Review:

Enterohemorrhagic *Escherichia coli* – Its Control from a Viewpoint of Food Safety –

Hiroshi Asakura, Yoshika Momose, and Fumiko Kasuga

Division of Biomedical Food Research, National Institute of Health Sciences
1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan.
E-mail: hasakura@nihs.go.jp
[Received January 28, 2011; accepted April 16, 2011]

This review focuses on the bacteriological nature and epidemics of enterohemorrhagic *Escherichia coli* (EHEC), a global scourge, from the viewpoint of food safety. Many human EHEC infections are linked to eating undercooked food and untreated water. We are still struggling to control this pathogen in the food chain, so we discuss current knowledge on sources of infection and EHEC distribution and survival mechanisms in foreign environments including the food matrix. We also introduce ways to effectively prevent food-borne EHEC infection.

Keywords: enterohemorrhagic *Escherichia coli* (EHEC), foodborne outbreak, food control

1. General Features of Enterohemorrhagic *Escherichia coli* (EHEC)

1.1. EHEC Defined

Escherichia coli is a Gram-negative rod-shaped facultative bacterium. Most *E. coli* strains harmlessly colonize the gastrointestinal tract of humans and animals as normal flora, but some strains have become pathogenic by acquiring virulence factors through plasmids, transposons, bacteriophages, and/or pathogenicity islands. These pathogenic strains are designated diarrheagenic *E. coli* and further categorized based on serogroups, pathogenicity mechanisms, clinical symptoms, and virulence factors [1, 2] into enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroadherent *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). Of these, EHEC is defined as producing Shiga toxin (Stx)– also called Shiga toxin-producing *E. coli* (STEC). *E. coli*, including EHEC, is classified by serotyping with somatic (O) antigens and flagella (H) antigens. Increasing our understanding of EHEC outbreak epidemiology, serotypes O26:H11, O91:H21, O111:H8, O157:NM, and O157:H7 have been recognized as frequently associated with human EHEC infection [3, 4]. *E. coli* O157:H7 is the EHEC serotype most frequently isolated from diarrhea subjects in the United States [5], Japan [6-8], and Europe [9]. In Japan, the dominant serotype associated with human STEC infection reported during 2007-2009 is O157:H7, followed by O26:H11 and O111:H−. These three account for over 82% of human STEC infection [6-8]. In 2009, the serotype O121 infection incidence increased, exceeding that of O111 due to a nursery school outbreak [8]. EHEC O157:H7 recognition has been facilitated by the availability of microbiological diagnostics based on the typical phenotypic feature of this pathogen, particularly its inability to be fermented in sorbitol in overnight incubation [10]. Some EHEC serotype O157:H− (nonmotile) strains and other serotypes do ferment rapidly in sorbitol and have increasingly emerged as important causes of human diseases [11]. Attention should be paid so that such strains are not missed in diagnostic procedures recommended for detecting *E. coli* O157:H7.

1.2. EHEC Infection History and Records

EHEC O157 was first recognized in 1982 as a human pathogen associated with outbreaks of bloody diarrhea in Oregon and Michigan, U.S.A. [12, 13], and also linked to sporadic cases of hemolytic uremic syndrome (HUS) in 1983 [14]. Since then, many EHEC-associated outbreaks have been reported in many developed countries, with EHEC O157 becoming to be well-recognized. Outbreak surveillance data reported by the U.S. Centers for Disease Control and Prevention (CDC) noted that EHEC O157 infection has decreased after peaking in 1999, with large outbreaks and sporadic cases continuing to occur.

In Japan, the first EHEC outbreak – EHEC O145:H− – was reported in 1984 at a Tokyo elementary school and the first reported fatal case due to EHEC infection occurred in Ehime in 1987 [15]. An EHEC O157 outbreak at a Saitama kindergarten in 1990 killed 2 children. Well water was specified as the causative agent [16]. Over 10,000 subjects suffered from EHEC O157 infection in 1996 nationwide in Japan [17]. Of these, thousands of children became involved due to outbreaks due to school lunches. Since then, 10 to 30 food-borne EHEC outbreaks have been reported annually [18]. In 2011, a multiprefectural *E. coli* O111 outbreak was occurred due to the intake of contaminated beef products. While non-O157 serogroups are clinically important, especially in young populations [8, 19], this review focuses on EHEC O157, globally the most prevalent serotype.

2. Clinical EHEC Infection Epidemics

EHEC O157 is recognized as a major public health concern in most developed countries. Although overall incidence of EHEC O157 is lower than that of other foodborne enteric pathogens, such as *Salmonella* or *Campylobacter* spp., disease due to EHEC O157 showed a much higher rate of hospitalization and mortality [20]. Human infection due to EHEC O157 presents as a broad clinical spectrum ranging from asymptomatic cases to death. Throughout epidemiological surveillance, this pathogen establishes infection at low doses – less than 10^2 per infection) [21]. After 2-9 days – usually 2-5 days – of incubation, most cases initiate nonbloody diarrhea and resolve without further complications. Some subjects, however, progress to bloody diarrhea or hemorrhagic colitis (HC) in 1-3 days. In 5-10% of HC subjects, the disease progresses to life-threatening sequelae, HUS, or thrombocytopenic purpura (TTP) [22]. Children and older persons are at increased risk for severe clinical symptoms such as HUS. Among therapy strategies studied are the use of antimicrobial agents and vaccination. Japan Ministry of Health, Labour, and Welfare (MHLW) has recommended using antimicrobial agents to treat EHEC infection [23], and the early use of antimicrobial agents has reduced HUS risk [24].The use of antimicrobial agents for treating EHEC, however, remains controversial.

The CDC estimated that EHEC O157 has cause 73,000 cases of illness, 2,200 hospitalizations, and 60 deaths annually in the U.S. [20].

3. Defining EHEC Pathogenesis Virulence Factors and Mechanisms

Stx production is considered essential, but not solely responsible for, disease development. EHEC O157 associated with severe human disease must colonize the intestinal mucosa. The presence of plasmid O157 (pO157) also correlates with the cause, as explained below.

3.1. Shiga Toxin (Stx)

The Stx conserved structure consists of one enzymatically active A subunit and five identical receptor-binding B subunits. B subunits bind to specific host receptors globotriaosylceramide (Gb3) or globotetraosylceramide (Gb4) [2]. Once binding is completed, the A-subunit is internalized into cytoplasm, thereby inhibiting host cell protein synthesis by specifically removing a single adenine residue from 28S rRNA of the 60S ribosomal subunit [25]. *Stx* genes are bacteriophage-encoded, contributing to bacterial evolution through its transmission [26]. Stx is divided into Stx1 and Stx2, each of which is further divided into variants based on nucleotide differences [27]. Stx1 is identical to Stx from *Shigella dysenteriae*. Stx2 is more often associated with HC or HUS in human infection than are Stx1-producing strains [28, 29]. Fujii et al. reported, however, that intravenous Stx1 was more lethal than Stx2 in the rabbit [30]. IT thus remains unclear if Stx2 is really more toxic than Stx1 or Stx2 is more produced by EHEC in the human intestine. Several Stx variants are known to associate with isolate sources [31].

3.2. Locus of Enterocyte Effacement

In addition to Stx production, a major virulence determinant is EHEC colonization capacity. After ingestion, EHEC bacteria colonize the surface of large-bowel enterocytes – typical of attaching and effacing (A/E) pathology mediated by type III secretion apparatus components encoded by the locus of enterocyte effacement (LEE) pathogenicity island [27]. This A/E lesion pathologically features microvilli effacement and bacterial adherence to epithelial cell membranes. Attached bacteria stimulate host cell actin polymerization accumulation, raising the attachment pedestal [32]. Enteropathogenic *E. coli* (EPEC) also conserves the LEE and LEE presence is strongly disease-associated [33]. The LEE consists of at least 41 different genes organized into three major regions – (i) a type III secretion system (TTSS) exporting effecter molecules, (ii) an adhesion called intimin and its translocated receptor Tir translocated into the host cell membrane by the TTSS, and (iii) several secreted proteins (Esp) as a part of TTSS important in modifying host cell signal transduction while A/E lesions form [34, 35]. Non-LEE encoded effectors have been identified, and clarifying their roles is expected to increase understanding of EHEC infection pathogenesis [36].

3.3. Plasmid O157 (pO157)

In addition to Stxs and the LEE, both chromosomally encoded, most clinical EHEC O157 isolates have a putative virulence plasmid called pO157. pO157, a nonconjugative F-like plasmid ranging from 92 to 104 kb in size, has a dynamic structure and includes different mobile genetic elements such as transposons, prophages, insertion sequences (IS), and parts of other plasmids. pO157 may facilitate antibiotic resistance gene transmission [37]. Study results on this plasmid's function in pathogenecity remain controversial.

3.4. Enterohemolysin (ehx)

Enterohemolysin, a repeat in toxin (RTX) member, was the first pO157 virulence factor described [38, 39]. The hemolysin operon *ehxCABD* may have a foreign origin as suggested by its different G+C% and codon use other than surrounding genetic contents. Several studies have shown that hemolysin is highly conserved among different EHEC serotypes, such as O157:H7, O111:H8, and O8:H19, but it remains unknown whether these exhibit identical biological activity [40].

3.5. Catalase-Peroxidase (KatP)

The *katP* gene product has catalase-peroxidase activity highly homologous to bacterial bifunctional catalase peroxidase [41]. The *katP* gene, found in all EHEC O157

strains, is not found in other pathogenic *E. coli,* including EPEC, ETEC, EIEC, and EAEC. The N-terminal signal sequence suggests this enzyme is transported through the cytoplasmic membrane and reduces oxidative stress to help use the by-product oxygen under diminished or deprived oxygen conditions in the host intestine, which may facilitate colonizing EHEC O157 bacteria in host intestinal tracts.

3.6. Adhesins and Others

A number of EHEC able to cause HUS have been characterized as LEE-negative strains [42], so this island is not essential to pathogenesis. Indeed, additional virulence factors encoded outside the LEE have been identified and proposed as be putative adhesins (adherence factors). These include the enterohemorrhagic *E. coli* factor for adherence (Efa1) [43]; Iha, an adherence-conferring protein similar to the *Vibrio cholerae* IrgA [44]; Saa, an autoagglutinating adhesin identified in LEE-negative EHEC serotype O113:H21 [45]; a long polar fimbriae (Lpf) closely related to Lpf of *Salmonella enterica* serovar Typhimurium [46]; and a protein, ToxB, required to fully express adherence in the O157:H7 Sakai strain [47].

The presence of other toxins possibly playing a role in STEC pathogenesis has been reported. CDT-V, a new cytolethal distending toxin (CDT) family member, was identified in O157 and particular non-O157 STEC serotypes [48, 49]. Bielaszewska et al. (2005) showed that the CDT-V could irreversibly damage human endothelial cells [50], suggesting that this toxin may contribute to STEC-mediated diseases – particularly in those due to LEE-negative strains. Subtilase cytotoxin (SubAB) is the prototype of a recently discovered AB5 cytotoxin produced by some STEC isolated from cases of severe human disease [51]. Intraperitoneal injection of SubAB causes microangiopathic hemolytic anemia, thrombocytopenia, and renal impairment in mice – typical of Shiga toxin-induced HUS [52].

Other virulence factors such as the type II secretion system (T2SS), serine protease EspP [53], metalloprotease [54], and ToxB, have also been reported to affect EHEC O157 capacity in intestinal colonization. Similar to EPEC, most EHEC O157 isolates have other type III secretion machinery encoded by *E. coli* type III secretion 2 (ETT2) loci mainly consisting of *epr*, *epa*, and *eiv* genes [55].

4. EHEC Transmission Routes

Mounting evidence suggests that EHEC bacteria are distributed in a variety of environments, including wildlife and farm animals, water, dairy and meat foods, and soil.

The most frequent human EHEC infection transmission route is thought to be consuming contaminated food and water [56]. It can also be transmitted, however, from person to person, particularly at child day-care facilities and homes for the elderly, and also by direct contact with an animal reservoir. Of the 350 outbreaks reported to the CDC from 1982 to 2002, the EHEC transmission routes determined were food-borne (52%), unknown (21%), person-to-person (14%), waterborne (9%), and animal contact (3%) [56].

During surveillance in 1995-6 in the UK, EHEC O157 was isolated from 15.7% of cattle, with a monthly prevalence from 5% to 37%. EHEC O157 was also isolated from 2.2% of sheep. During surveillance in 1996, EHEC O157 was isolated from 5.9% of samples of lamb products and from 1.5% of samples of beef products, despite the much higher prevalence in cattle than in sheep [57]. Other hosts of EHEC O157 [58-63] have included pigs, sheep, goats, seagulls, flies, dogs, and cats.

Contaminated beef products are common vehicles for EHEC infection after becoming contaminated during slaughter. The process of making ground beef may facilitate this pathogen's internalization from the meat surface. Such inadequate beef carcass processing lets bacteria survive and spread. Contaminated food other than ground beef linked to EHEC infection includes unpasteurized milk, unclean drinking water, salami, beef jerky, and cattle offal and fresh produce such as lettuce, radish sprouts, fresh spinach, and apple cider. The largest EHEC O157 outbreak was traced to radish sprout contamination (1996) in Osaka, Japan, where 7,966 individuals were diagnosed with confirmed infection [17]. Epidemiological studies indicate that these food products appear to have been contaminated through bovine fecal matter. Prevention in cattle is likely one of the best ways to control EHEC. Proposals for doing so on the farm have included improving cattle management, identifying inhibitory feed, implementing immunization, currently used feed additives, and using probiotic cultures [64, 65].

5. Cattle as an Important Human EHEC Reservoir

Most hosts causing asymptomatic EHEC become potential human-infection reservoirs. Cattle are the natural reservoir of EHEC O157, which has, if rarely caused diarrhea in young calves with this pathogen. Epidemiological data shows that between 1% and 50% of healthy cattle carry and shed EHEC O157 in their feces at any given time [66-68]. In ruminants, EHEC O157 is naturally colonized at the lymphoid follicle-dense mucosa at the terminal rectum, i.e., rectoanal junction mucosa, known as a principal colonization site in cattle [69, 70]. Colonization at this site requires that bacteria pass through the gastric rumen, which contains gastric juice. EHEC O157 develops sophisticated resistance mechanisms against acidic pH in rumen gastric juice [71, 72], facilitating colonization and long-term pathogen persistence in the ruminant gut.

Cattle excreting more EHEC O157 than others are known as super-shedders [52] – a phenomenon having important consequences in EHEC O157 epidemiology in cattle – its main reservoir – and for human infection

risk, particularly in environmental exposure. Control ultimately targeting super-shedders may prove highly effective [73]. Menrath *et al.* (2010) noted that the presence of non-O157 EHEC bovine super-shedders in a herd created significant risk of human EHEC infection [74].

6. Prevalence and Survival in Hostile Environments

6.1. EHEC Prevalence in Food Products

EHEC isolation principally relies on culture enrichment. In combination with this golden standard method, molecular biology advances in the last decade has improved our knowledge of pathogen prevalence in hostile environments.

6.1.1. Meat Products

Meat products are often contaminated with EHEC O157 at slaughter when the hide or gastrointestinal tract is removed [75, 76]. According to literature surveyed by Hussein and Bollinger [77], EHEC O157 prevalence in meat products ranged from 0.01 to 43.4% in packing plants, 0.1 to 54.2% in supermarkets, and an average of 2.4% in fast-food restaurants. Regarding product type, EHEC O157 contamination ranged from 0.1% to 54.2% in ground beef, 0.1% to 4.4% in sausage, 1.1% to 36.0% in unspecified retail cuts, and 0.01% to 43.4% in whole carcasses [77].

6.1.2. Dairy Products

EHEC-associated outbreaks have been attributed to dairy product consumption, including raw milk [78-80], cheeses [81-84], and yogurt [85]. A recent study based on genetic screening showed higher EHEC prevalence in soft cheese than that in semi-hard and hard cheese products – 5.4% vs. 10.0% [86]. Despite of O157 outbreaks due to raw milk outside of Japan, unpasteurized milk is not usually domestically distributed and would not seem a major infection source.

Human outbreaks due to EHEC-contaminated cheese in the US, Canada, and European countries showed that soft cheese such as Camembert and Brie posed a greater EHEC transmission risk than other cheese [87]. A trace study of nonpathogenic *E. coli* prevalence in the cheese manufacturing process showed that contamination occurs at certain points in cheese production and processing [88-90].

6.1.3. Vegetables

Although EHEC infection has been linked to beef more often than to any other food product, serious outbreaks have been traced to the consumption of contaminated vegetables, including radish sprouts and prepackaged spinach. Japan's largest EHEC O157 outbreak in 1996 was attributed to radish sprouts [91]. Instant "asazuke" pickles also apparently caused a Saitama prefecture O157 outbreak in 2001 [92]. In the US, spinach products were identified as causative in a multistate outbreak in 2006 [93]. Lettuce leaves were associated with large EHEC O157 outbreaks in Sweden [94], the Netherlands, and Iceland [95]. It is likely that EHEC O157 binds to lettuce leaves by the mechanisms involving filamentous type III secretion, flagella, and curli fimbriae [96, 97]. Transmission routes to such fresh produce remain only partly understood. With these foods often consumed without heating, further epidemiological study is required before this pathogen could be controlled in the food chain.

6.2. EHEC O157 Survival in Food and Environments

EHEC survives and persists in numerous soil, water, food, and animal reservoir environments. This pathogen has been shown to survive a year after excretion in manure-treated soil and for 21 months in raw non-composted manure [73]. Composting manure effectively destroys EHEC O157 if temperature is maintained above 50°C for 6 days [98]. In water environment, EHEC O157 also survives long periods, especially at cold temperatures. Water trough sediment contaminated by bovine feces serves as a long-term – 8 months or more – reservoir of EHEC O157. Bacteria surviving in contaminated troughs can be a source of infection [99]. The first EHEC O157 outbreak in Japan was reported in 1990, originated in a water-borne source [16].

EHEC O157 bacteria survive in several types of foods, although they include a variety of foreign stresses. EHEC O157 ability to survive outside the reservoir host gut is linked to increased risk of human infection. To survive in different environments, EHEC O157 need only adapt to variations or extreme changes in temperature, pH, and osmolarity common in nature. It is now clear that exopolysaccharide (EPS) production contributes to tolerance of heat and acid stress, and the alteration of lipid composition in membranes is induced by heat stress [100]. It remains unclear, however, how, in order to survive, bacteria sense environmental stimuli to overproduce EPS.

Evidence is mounting that, in a survival bid under harsh conditions, bacteria enter viable but nonculturable (VBNC) status [101], defined as loss of culturability, while retaining metabolic activity such as DNA stability and membrane integrity [101]. VBNC status in pathogenic bacteria is a potential human infection risk factor because such dormancy enables bacteria to replicate and cause infection after being resuscitated from dormancy [102]. We previously showed that EHEC O157 cells survive in salted salmon roe by entering into VBNC status [103]. Subsequent analysis showed that VBNC-status EHEC O157 is resuscitated to replicate by pre-enrichment under low nutrient conditions in the presence of antioxidants [103]. Throughout proteomic analysis, we found that outer membrane protein W (OmpW) expression corresponds to VBNC occurrence in this pathogen [104]. In-depth analysis assessing VBNC impact on pub-

lic health risk is needed to clarify the public health impact of VBNC bacteria. In any case, we must consider the presence of such dormant bacteria in microbial testing using molecular techniques.

7. EHEC Control for Food Safety in Japan

After tragic sequential EHEC O157 outbreaks in 1996 in Japanese school kitchens that killed children, Japan's MHLW and Ministry of Education, Culture, Sport, Science, and Technology (MEXT) have taken measures to improve cooking facilities hygiene, as outlined below.

7.1. Genetic EHEC Analysis for Efficiently Detecting Multiprefectural Outbreaks

According to the Law on Infectious Disease Prevention and Medical Care of Infected Subjects, also known as the Infectious Disease Control Law, enacted in April 1999, EHEC was classified in the National Epidemiological Surveillance of Infectious Diseases (NESID) as a category III notifiable infectious disease. Physicians must immediately report diagnosing EHEC based on its isolation and detection of Shiga toxin (Stx). Local governments also report and investigate EHEC as a form of food poisoning. Such reports are submitted to and compiled by the MHLW under the Food Sanitation Law [8].

Prefectures and legally-designated cities have public health institutes (PHIs) that isolate and study EHEC, e.g., serotyping and Stx-typing, and send isolates to the National Institute of Infectious Diseases (NIID) Department of Bacteriology for molecular epidemiological analysis. Pulsed-field gel electrophoresis (PFGE) and, for some isolates, multiple-locus variable-number tandem repeat analysis (MLVA), are applied to compare isolates [105, 106]. If analyzed genomic patterns are indistinguishable among bacterial strains isolated in different, distant areas, a common infection source such as widely distributed food is suspected. Detecting such genomic clusters helps identifying multiprefecture outbreaks, including diffuse outbreaks and efficient investigation and prevention of other illnesses. In 2009, the NIID Department of Bacteriology analyzed 2,243 EHEC isolates, demonstrating three identical genetic clusters for isolates from subjects in diffuse outbreaks occurring in steak and barbecue restaurant chains, as detailed in PulseNet Japan [107].

7.2. Food-Safety Manuals for Large-Scale Cooking Facilities

The MHLW is responsible for food-safety in general, including hygienic control of commercial cooking facilities and kitchens at child-care and social-welfare institutions. The MHLW issued new food-safety manuals for these facilities introducing the hazard analysis and critical control point (HACCP) concept, including time and temperature control, and strongly recommending kitchens to keep records as detailed in the manuals. For large-scale cooking facilities preparing over 750 meals per day or 300 dishes at a time for a single menu, the manuals advised saving at 50 gram portions of individual raw foods and cooked dishes for at least 2 weeks at temperatures below −20°C. Although this recommendation is not mandatory, most large kitchens introduced this, which also came into use at schools, daycare centers, hospitals, and other child-care and social-welfare facilities [108].

Food storage has helped identify causative food and agents from outbreaks. Thanks to microbiologically good conditions of food materials kept in freezers, it has often been possible to detail causative pathogen in food, greatly facilitating microbiological risk assessment, as detailed below.

The number of reported EHEC O157 outbreaks significantly decreased in the late 90s, but has remained essentially unchanged recently. To make matters worse, Infectious Disease Control Law statistics indicates 3,000 to 4,000 EHEC cases occur annually, as stated [8]. This means a rather steady level of O157 infection, most of which is sporadic, among a Japanese population with unknown infection routes and causes. This also suggests the need for other control measures preventing O157.

7.3. Food-Safety Manual for School Kitchens

Under the strong influence of the 1996 school lunch outbreaks, MEXT issued a new manual for school kitchen food hygiene and organized a task force of microbiological experts to inspect school kitchens. The manual differs from that by the MHLW, along with kitchen organization and cooking procedures, and does not at first focus on the HACCP concept, focusing, instead, on detailed daily and monthly checklist use and other documentation. Prefectures and city boards of education have cooperated with MEXT and individual facilities.

Once an outbreak occurs at a school kitchen, a task force inspection team is sent to work with local health centers on investigating the facility and cooking procedures throughout the process, interviewing school personnel and responsible board of education members. Their reports are summarized annually with microbiological examination results for food and environmental samples from facilities and visual explanations of advantageous and disadvantageous practices used as educational tools for individuals involved in school lunches.

With such practical instructions and training, infectious outbreaks due to school lunches dramatically decreased to where no outbreaks occurred at all in 2010.

7.4. Microbiological Food-Safety Risk Analysis

The United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have been working jointly for decades on different aspects of food safety. Under the Joint FAO/WHO Food Standards Programme, the Codex Alimentarius Commission (CAC) was set up in 1963 to develop food standards, guidelines, and other texts, including codes of practice [109]. In the mid 1990s, the CAC initiated discussions introducing a risk analysis framework to improve global

food safety. Risk analysis, a new food safety concept at the, was viewed by the CAC, with help from FAO/WHO expert committees, in a series of guidelines to three risk analysis components, i.e., risk management, risk assessment and risk communication.

These components involve logical determination and evaluation of risk based on scientific evidence in risk assessment. Scientific procedures taking over 40 years to establish control levels for food additives and pesticide residues are regarded as risk assessment for chemicals. No global experience had been gained, however, on microbiological risk assessment (MRA)., so the CAC asked FAO/WHO to set up a new expert meeting body, the Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (JEMRA). JEMRA initiated model risk assessments of prioritized pathogens and developed detailed guidelines for conducting MRA [110]. JEMRA has also published 3 guidelines and 7 risk-assessment documents, including a meeting report of *E. coli* in meat and meat products [111].

MRA estimates risk and predicts risk reduction for possible mitigation measures not obtainable in previous microbiological approaches. By conducting MRA, a government can compare the effectiveness of multiple control measures conceivably applied at different points in the food chain, and it is even possible to evaluate the equivalency of food-safety standards between food exporting and importing countries. Individual countries have started their own risk assessment. EHEC is one of the important targets of MRA [112-116], so the Food Safety Commission, Japan's food-safety risk assessment organization, has developed a risk profile for EHEC [21].

Numerous scientific articles have been published proposing new data to be used and technical methods of data analysis for MRA. Two of these papers [117, 118] used Japanese outbreak data that showed the dose of EHEC ingested by subjects and the ratio of ill people (attack rate) in the exposed population. These datasets are critical for inducing the dose-response relationship of a pathogen for estimating the probability of illness occurrence with a given dose of ingested pathogens. This, in turn, is used to estimate total risk based on the exposure level of a pathogen in a target population. Such datasets are usually difficult to obtain from outbreak investigations because causative pathogens are not always possible to detect from leftover food and, even if detected, enumerating the pathogen is not meaningful when food conditions have greatly changed microbiological food specimen contents. Food storage has contributed significantly to overcoming this difficulty, as stated, and Japanese outbreak data enabled better dose response models for EHEC to be developed.

Acknowledgements

This work was financially supported in part by a grant from the MHLW Research on Food Safety, Japan, and by grants-in-aid from the Food Safety Commission, Japan (No. 0805).

References:

[1] J. B. Kaper, J. P. Nataro, and H. L. Mobley, "Pathogenic *Escherichia coli*," Nat. Rev. Microbiol. 2, pp. 123-140, 2004.
[2] J. P. Nataro and J. B. Kaper, "Diarrheagenic *Escherichia coli*," Clin. Microbiol. Rev.11, pp. 142-201, 1998.
[3] A. R. Melton-Celsa, S. C. Darnell, and A. D. O'Brien, "Activation of Shiga-Like Toxins by Mouse and Human Intestinal Mucus Correlates with Virulence of Enterohemorrhagic *Escherichia coli* O91:H21 Isolates in Orally Infected, Streptomycin-Treated Mice," Infect. Immun. 64, pp. 1569-1576, 1996.
[4] A. W. Paton and J. C. Paton, "Direct Detection of Shiga Toxigenic *Escherichia coli* Strains Belonging to Serogroups O111, O157, and O113 by Multiplex PCR," J. Clin. Microbiol. 37, pp. 3362-3365, 1999.
[5] CDC. OutbreakNet. Foodborne outbreak online database, http://wwwn.cdc.gov/foodborneoutbreaks/Default.aspx [accessed at Dec. 20, 2010]
[6] NIID and MHLW, "Enterohemorrhagic *Escherichia coli* Infection in Japan as of April 2008," Infectious Agents Surveillance Report (IASR), 29, pp. 117-118, 2008.
[7] NIID and MHLW, "Enterohemorrhagic *Escherichia coli* Infection in Japan as of April 2009," Infectious Agents Surveillance Report (IASR), 30, pp. 119-120. 2009.
[8] NIID and MHLW, "Enterohemorrhagic *Escherichia coli* Infection in Japan as of May 2010," Infectious Agents Surveillance Report (IASR), 31, pp. 152-154, 2010.
[9] European Food Safety Authority, "The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-Borne Outbreaks in the European Union in 2008," EFSA J., pp. 1496, 2010.
[10] S. B. March and S. Ratnam, "Sorbitol-MacConkey Medium for Detection of *Escherichia coli* O157:H7 Associated with Hemorrhagic Colitis," J. Clin. Microbiol. 23, pp. 869-872, 1986.
[11] H. Karch and M. Bielaszewska, "Sorbitol-Fermenting Shiga Toxin-Producing *Escherichia coli* O157:H(-) Strains: Epidemiology, Phenotypic and Molecular Characteristics, and Microbiological Diagnosis," J. Clin. Microbiol. 39, pp. 2043-2049, 2001.
[12] L. W. Riley, R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, et al, "Hemorrhagic Colitis Associated with a Rare *Escherichia coli* Serotype," N. Engl. J. Med. 308, pp. 681-685, 1983.
[13] J. G. Wells, B. R. Davis, I. K. Wachsmuth, L. W. Riley, R. S. Remis, R. Sokolow, and G. K. Morris, "Laboratory Investigation of Hemorrhagic Colitis Outbreaks Associated with a Rare *Escherichia coli* Serotype," J. Clin. Microbiol. 18, pp. 512-520, 1983.
[14] M. A. Karmali, B. T. Steele, M. Petric, and C. Lim, "Sporadic Cases of Haemolytic-Uraemic Syndrome Associated with Faecal Cytotoxin and Cytotoxin-Producing *Escherichia coli* in Stools," Lancet. 1, pp. 619-620, 1983.
[15] S. Yamasaki and Y. Takeda, "Enterohemorrhagic *Escherichia coli* O157:H7 Episode in Japan with a Perspective on Vero Toxins (Shiga-Like Toxins)," J. Toxicol. Toxin Rev.16, pp. 229-240, 1997.
[16] K. Joh, "Clinical Microbiology," 23, pp. 869-878. 1996.
[17] H. Michino, K. Araki, S. Minami, S. Takaya, N. Sakai, M. Miyazaki, A. Ono, and H. Yanagawa, "Massive Outbreak of *Escherichia coli* O157:H7 Infection in School Children in Sakai City, Japan, Associated with Consumption of White Radish Sprouts," Am. J. Epidemiol. 150, pp. 787-796, 1999.
[18] Japan Ministry of Health, Labour, and Welfare, "Food Poisoning statistics," http://www.mhlw.go.jp/topics/ syokuchu/04.html#4-2 [accessed at 25, Jan., 2011]
[19] J. T. Brooks, E. G. Sowers, J. G. Wells, K. D. Greene, P. M. Griffin, R. M. Hoekstra, and N. A. Strockbine, "Non-O157 Shiga Toxin-Producing *Escherichia coli* Infections in the United States, 1983-2002," J. Infect. Dis. 192, pp. 1422-1429, 2005.
[20] P. S. Mead, L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe, "Food-Related Illness and Death in the United States," Emerg. Infect. Dis. 5, pp. 607-625, 1999.
[21] Japan Food safety Commission, "Risk Profile of Enterohemorrhagic *Escherichia coli* in Beef Products," 2006 (in Japanese), http://www.fsc.go.jp/senmon/biseibutu/risk_profile/enterohemorrhagic.pdf
[22] N. Banatvala, P. M. Griffin, K. D. Greene, T. J. Barrett, W. F. Bibb, J. H. Green, and J. G. Wells, "The United States National Prospective Hemolytic Uremic Syndrome Study: Microbiologic, Serologic, Clinical, and Epidemiologic Findings," J. Infect. Dis. 183, pp. 1063-1070, 2001.
[23] Japan Ministry of Health, Labour, and Welfare, "Guideline for the Treatment of Enterohemorrhagic *Escherichia coli* Infection," 1997.
[24] T. Takeda, K. Yoshino, H. Uchida, N. Ikeda, and M. Tanimura, "*Escherichia coli* O157:H7 and Other Shiga Toxin-Producing *E. coli* Strains," pp. 385-387, In J. B. Kaper, and A. D. O'Brien (ed.), "*Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* Strains," American Society for Microbiology, Washington, D.C. 1998.

[25] Y. Endo, K. Tsurugi, T. Yutsudo, Y. Takeda, T. Ogasawara, and K. Igarashi, "Site of Action of a Vero Toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga Toxin on Eukaryotic Ribosomes. RNA N-Glycosidase Activity of the Toxins," Eur. J. Biochem. 171, pp. 45-50, 1988.

[26] H. E. Allison, "Stx-Phages: Drivers and Mediators of the Evolution of STEC and STEC-Like Pathogens," Future Microbiol. 2, pp. 165-74, 2007.

[27] M. A. Karmali, "Infection by Shiga Toxin-Producing *Escherichia coli*: an Overview," Mol. Biotechnol, 26, pp. 117-122, 2004.

[28] P. Boerlin, S. A. McEwen, F. Boerlin-Petzold, J. B. Wilson, R. P. Johnson, and C. L. Gyles, "Associations Between Virulence Factors of Shiga Toxin-Producing *Escherichia coli* and Disease in Humans," J. Clin. Microbiol. 37, pp. 497-503, 1999.

[29] S. M. Ostroff, P. I. Tarr, M. A. Neill, J. H. Lewis, N. Hargrett-Bean, and J. M. Kobayashi, "Toxin Genotypes and Plasmid Profiles as Determinants of Systemic Sequelae in *Escherichia coli* O157:H7 Infections," J. Infect. Dis. 160, pp. 994-998, 1989.

[30] J. Fujii, Y. Kinoshita, T. Yutsudo, H. Taniguchi, T. Obrig, and S. Yoshida, "Toxicity of Shiga Toxin 1 in the Central Nervous System of Rabbits," Infect. Immun. 69, pp. 6545-6548, 2001.

[31] H. Asakura, S. Makino, H. Kobori, M. Watarai, T. Shirahata, T. Ikeda, K. Takeshi, "Phylogenetic Diversity and Similarity of Active Sites of Shiga Toxin (Stx) in Shiga Toxin-Producing *Escherichia coli* (STEC) Isolates from Humans and Animals. Epidemiol Infect," 127, pp. 27-36, 2001.

[32] A. Caprioli, S. Morabito, H. Brugère, and E. Oswald, "Enterohaemorrhagic *Escherichia coli*: Emerging Issues on Virulence and Modes of Transmission," Vet. Res. 36, pp. 289-311, 2005.

[33] P. M. Griffin and R. V. Tauxe, "The Epidemiology of Infections Caused by *Escherichia coli* O157:H7, Other Enterohemorrhagic *E. coli*, and the Associated Hemolytic Uremic Syndrome," Epidemiol. Rev.13, pp. 60-98, 1991.

[34] R. M. Delahay, G. Frankel, and S. Knutton, "Intimate Interactions of Enteropathogenic *Escherichia coli* at the Host Cell Surface," Curr. Opin. Infect. Dis. 14, pp. 559-565, 2001.

[35] N. T. Perna, G. F. Mayhew, G. Posfai, S. Elliott, M. S. Donnenberg, J. B. Kaper, and F. R. Blattner, "Molecular Evolution of a Pathogenicity Island from Enterohemorrhagic *Escherichia coli* O157:H7," Infect. Immun. 66, pp. 3810-3817, 1998.

[36] W. Deng, J. L. Puente, S. Gruenheid, Y. Li, B. A. Vallance, A. Vázquez, et al, "Dissecting Virulence: Systematic and Functional Analyses of a Pathogenicity Island," Proc. Natl. Acad. Sci. U.S.A. 101, pp. 3597-3602, 2004.

[37] S. Makino, H. Asakura, T. Obayashi, T. Shirahata, T. Ikeda, K. Takeshi, "Molecular Epidemiological Study on Tetracycline Resistance R Plasmids in Enterohaemorrhagic *Escherichia coli* O157:H7," Epidemiol. Infect. 123, pp. 25-30, 1999.

[38] M. E. Bauer and R. A. Welch, "Characterization of an RTX Toxin from Enterohemorrhagic *Escherichia coli* O157:H7," Infect. Immun. 64, pp. 167-175, 1996.

[39] H. Schmidt, L. Beutin, and H. Karch, "Molecular Analysis of the Plasmid-Encoded Hemolysin of *Escherichia coli* O157:H7 Strain EDL 933," Infect. Immun. 63, pp. 1055-1061, 1995.

[40] M. M. Brashears, M. L. Galyean, G. H. Loneragan, J. E. Mann, and K. Killinger-Mann, "Prevalence of *Escherichia coli* O157:H7 and Performance by Beef Feedlot Cattle Given Lactobacillus Direct-Fed Microbials," J. Food Prot. 66, pp. 748-754, 2003.

[41] W. Brunder, H. Schmidt, and H. Karch, "KatP, a Novel Catalase-Peroxidase Encoded by the Large Plasmid of Enterohaemorrhagic *Escherichia coli* 0157:H7," Microbiology 142, pp. 3305-3315, 1996.

[42] J. C. Paton and A. W. Paton, "Pathogenesis and Diagnosis of Shiga Toxin-Producing *Escherichia coli* Infections," Clin. Microbiol. Rev.11, pp. 450-479, 1998.

[43] L. Nicholls, T. H. Grant, and R. M. Robins-Browne, "Identification of a Novel Genetic Locus that is Required for in Vitro Adhesion of a Clinical Isolate of Enterohaemorrhagic *Escherichia coli* to Epithelial Cells," Mol. Microbiol. 35, pp. 275-288, 2000.

[44] P. I. Tarr, S. S. Bilge, J. C. Vary Jr, S. Jelacic, R. L. Habeeb, T. R. Ward, M. R. Baylor, and T. E. Besser, "Iha: a Novel *Escherichia coli* O157:H7 Adherence-Conferring Molecule Encoded on a Recently Acquired Chromosomal Island of Conserved Structure," Infect. Immun. 68, pp. 1400-1407, 2000.

[45] A. W. Paton, P. Srimanote, M. C. Woodrow, and J. C. Paton, "Characterization of Saa, a Novel Autoagglutinating Adhesin Produced by Locus of Enterocyte Effacement-Negative Shiga-Toxigenic *Escherichia coli* Strains that are Virulent for Humans," Infect. Immun. 69, pp. 6999-7009, 2001.

[46] S. Doughty, J. Sloan, V. Bennett-Wood, M. Robertson, R. M. Robins-Browne, and E. L. Hartland, "Identification of a Novel Fimbrial Gene Cluster Related to Long Polar Fimbriae in Locus of Enterocyte Effacement-Negative Strains of Enterohemorrhagic *Escherichia coli*," Infect. Immun. 70, pp. 6761-6769, 2002.

[47] I. Tatsuno, M. Horie, H. Abe, T. Miki, K. Makino, H. Shinagawa, H. Taguchi, S. Kamiya, T. Hayashi, and C. Sasakawa, "*toxB* Gene on pO157 of Enterohemorrhagic *Escherichia coli* O157:H7 is Required for Full Epithelial Cell Adherence Phenotype," Infect. Immun. 69, pp. 6660-6669, 2001.

[48] A. Janka, M. Bielaszewska, U. Dobrindt, L. Greune, M. A. Schmidt, and H. Karch, "Cytolethal Distending Toxin Gene Cluster in Enterohemorrhagic *Escherichia coli* O157:H– and O157:H7: Characterization and Evolutionary Considerations," Infect. Immun. 71, pp. 3634-3638. 2003.

[49] M. Bielaszewska, M. Fell, L. Greune, R. Prager, A. Fruth, H. Tschäpe, M. A. Schmidt, and H. Karch, "Characterization of Cytolethal Distending Toxin Genes and Expression in Shiga Toxin-Producing *Escherichia coli* Strains of Non-O157 Serogroups," Infect. Immun. 72, pp. 1812-1816, 2004.

[50] M. Bielaszewska, B. Sinha, T. Kuczius, and H. Karch, "Cytolethal Distending Toxin from Shiga Toxin-Producing *Escherichia coli* O157 Causes Irreversible G2/M Arrest, Inhibition of Proliferation, and Death of Human Endothelial Cells," Infect. Immun. 73, pp. 552-562, 2005.

[51] A. W. Paton and J. C. Paton, "*Escherichia coli* Subtilase Cytotoxin," Toxins (Basel), 2, pp. 215-228, 2010.

[52] H. Wang, J. C. Paton, and A. W. Paton, "Pathologic Changes in Mice Induced by Subtilase Cytotoxin, a Potent New *Escherichia coli* AB5 Toxin that Targets the Endoplasmic Reticulum," J. Infect. Dis. 196, pp. 1093-1101, 2007.

[53] W. Brunder, H. Schmidt, and H. Karch, "EspP, a Novel Extracellular Serine Protease of Enterohaemorrhagic *Escherichia coli* O157:H7 Cleaves Human Coagulation Factor V," Mol. Microbiol. 24, pp. 767-778, 1997.

[54] W. W. Lathem, T. E. Grys, S. E. Witowski, A. G. Torres, J. B. Kaper, P. I. Tarr, and R. A. Welch, "StcE, a Metalloprotease Secreted by *Escherichia coli* O157:H7, Specifically Cleaves C1 Esterase Inhibitor," Mol. Microbiol. 45, pp. 277-288, 2002.

[55] S. Makino, T. Tobe, H. Asakura, M. Watarai, T. Ikeda, K. Takeshi, and C. Sasakawa, "Distribution of the Secondary Type III Secretion System Locus Found in Enterohemorrhagic *Escherichia coli* O157:H7 Isolates Among Shiga Toxin-Producing *E. coli* Strains," J. Clin. Microbiol. 41, pp. 2341-2347, 2003.

[56] J. M. Rangel, P. H. Sparling, C. Crowe, P. M. Griffin, and D. L. Swerdlow, "Epidemiology of *Escherichia coli* O157:H7 Outbreaks, United States, 1982-2002," Emerg. Infect. Dis. 11, pp. 603-609, 2005.

[57] P. A. Chapman, "Sources of *Escherichia coli* O157 and Experiences Over the Past 15 Years in Sheffield, UK," Symp. Ser. Soc. Appl. Microbiol. 29, pp. 51S-60S, 2000.

[58] T. R. Callaway, R. C. Anderson, G. Tellez, C. Rosario, G. M. Nava, C. Eslava, M. A. Blanco, M. A. Quiroz, A. Olguín, M. Herradora, T. S. Edrington, K. J. Genovese, R. B. Harvey, and D. J. Nisbet, "Prevalence of *Escherichia coli* O157 in Cattle and Swine in Central Mexico," J. Food Prot. 67, pp. 2274-2276, 2004.

[59] A. E. Heuvelink, F. L. van den Biggelaar, E. de Boer, R. G. Herbes, W. J. Melchers, J. H. Huis in't Veld, and L. A. Monnens, "Isolation and Characterization of Verocytotoxin- Producing *Escherichia coli* O157 Strains from Dutch Cattle and Sheep," J. Clin. Microbiol. 36, pp. 878-882, 1998.

[60] M. Iwasa, S. Makino, H. Asakura, H. Kobori, and Y. Morimoto, "Detection of *Escherichia coli* O157:H7 from Musca domestica (Diptera: Muscidae) at a Cattle Farm in Japan," J. Med. Entomol. 36, pp. 108-112, 1999.

[61] S. Makino, H. Kobori, H. Asakura, M. Watarai, T. Shirahata, T. Ikeda, K. Takeshi, and T. Tsukamoto, "Detection and Characterization of Shiga Toxin-Producing *Escherichia coli* from Seagulls," Epidemiol. Infect. 125, pp. 55-61, 2000.

[62] G. Mersha, D. Asrat, B. M. Zewde, and M. Kyule, "Occurrence of *Escherichia coli* O157:H7 in Faeces, Skin and Carcasses from Sheep and Goats in Ethiopia," Lett. Appl. Microbiol. 50, pp. 71-76, 2010.

[63] Y. Kataoka, Y. Irie, T. Sawada, and M. Nakazawa, "A 3-Year Epidemiological Surveillance of *Escherichia coli* O157:H7 in Dogs and Cats in Japan," J. Vet. Med. Sci. 72, pp. 791-794, 2010.

[64] T. R. Callaway, R. C. Anderson, K. J. Genovese, T. L. Poole, T. J. Anderson, J. A. Byrd, L. F. Kubena, and D. J. Nisbet, "Sodium Chlorate Supplementation Reduces *E. coli* O157:H7 Population in Cattle," J. Anim. Sci. 80, pp. 1683-1689, 2002.

[65] A. Chesson, C. S. Stewart, and R. J. Wallace, "Influence of Plant Phenolic Acids on Growth and Cellulolytic Activity of Rumen Bacteria," Appl. Environ. Microbiol. 44, pp. 597-603, 1982.

[66] S. Cho, J. B. Bender, F. Diez-Gonzalez, C. P. Fossler, C. W. Hedberg, J. B. Kaneene, P. L. Ruegg, L. D. Warnick, and S. J. Wells, "Prevalence and Characterization of *Escherichia coli* O157 Isolates from Minnesota Dairy Farms and County Fairs," J. Food Prot. 69, pp. 252-259, 2006.

[67] J. R. Dunn, J. E. Keen, and R. A. Thompson, "Prevalence of Shiga-Toxigenic *Escherichia coli* O157:H7 in Adult Dairy Cattle," J. Am. Vet. Med. Assoc. 224, pp. 1151-1158, 2004.

[68] D. D. Hancock, T. E. Besser, D. H. Rice, D. E. Herriott, and P. I. Tarr, "A Longitudinal Study of *Escherichia coli* O157 in Fourteen Cattle Herds," Epidemiol. Infect. 118, pp. 193-195, 1997.

[69] J. Y. Lim, J. Li, H. Sheng, T. E. Besser, K. Potter, and C. J. Hovde, "*Escherichia coli* O157:H7 Colonization at the Rectoanal Junction of Long-Duration Culture-Positive Cattle," Appl. Environ. Microbiol. 73, pp. 1380-1382, 2007.

[70] S. W. Naylor, J. C. Low, T. E. Besser, A. Mahajan, G. J. Gunn, M. C. Pearce, I. J. McKendrick, D. G. E. Smith, and D. L. Gally, "Lymphoid Follicle-Dense Mucosa at the Terminal Rectum is the Principal Site of Colonization of Enterohemorrhagic *Escherichia coli* O157:H7 in the Bovine Host," Infect. Immun. 71, pp. 1505-1512, 2003.

[71] M. M. Benjamin and A. R. Datta, "Acid Tolerance of Enterohemorrhagic *Escherichia coli*," Appl. Environ. Microbiol. 61, pp. 1669-1672, 1995.

[72] M. P. Castanie-Cornet, T. A. Penfound, D. Smith, J. F. Elliott, and J. W. Foster, "Control of Acid Resistance in *Escherichia coli*," J. Bacteriol. 181, pp. 3525-3535, 1999.

[73] M. Chase-Topping, D. Gally, C. Low, L. Matthews, and M. Woolhouse, "Super-Shedding and the Link Between Human Infection and Livestock Carriage of *Escherichia coli* O157," Nat. Rev. Microbiol. 6, pp. 904-12, 2008.

[74] A. Menrath, L. H. Wieler, K. Heidemanns, T. Semmler, A. Fruth, N. Kemper, "Shiga Toxin Producing *Escherichia coli*: Identification of Non-O157:H7 Super-Shedding Cows and Related Risk Factors," Gut. Pathog. 2, e7, 2010.

[75] R. O. Elder, J. E. Keen, G. R. Siragusa, G. A. Barkocy-Gallagher, M. Koohmaraie, and W. W. Laegreid, "Correlation of Enterohemorrhagic *Escherichia coli* O157 Prevalence in Feces, Hides, and Carcasses of Beef Cattle During Processing," Proc. Natl. Acad. Sci. USA, 97, pp. 2999-3003, 2000.

[76] J. M. McEvoy, A. M. Doherty, J. J. Sheridan, F. M. Thomson-Carter, P. Garvey, L. McGuire, I. S. Blair, and D. A. McDowell, "The Prevalence and Spread of *Escherichia coli* O157:H7 at a Commercial Beef Abattoir," J. Appl. Microbiol. 95, pp. 256-266, 2003.

[77] H. S. Hussein and L. M. Bollinger, "Prevalence of Shiga Toxin Producing *Escherichia coli* in Beef," Meat Sci. 71, pp. 676-689, 2005.

[78] F. Allerberger, M. Wagner, P. Schweiger, H.-P. Rammer, A. Resch, M. P. Dierich, A. W. Friedrich, and H. Karch, "*Escherichia coli* O157 Infections and Unpasteurised Milk," Eurosurveillance. 6, pp. 147-151, 2001.

[79] E. Lahti, M. Eklund, P. Ruutu, A. Siitonrn, L. Rantala, P. Nuorti, and T. Honkanen-Buzalski, "Use of Phenotyping and Genotyping to Verify Transmission of *Escherichia coli* O157:H7 from Dairy Farms," Eur. J. Clin. Microbiol. Infect. Dis. 21, pp. 189-195, 2002.

[80] M. L. Martin, L. D. Shipman, J. G. Wells, M. E. Potter, K. Hedberg, I. K. Wachsmuth, R. V. Tauxe, J. P. Davis, J. Arnoldi, and J. Tilleli, "Isolation of *Escherichia coli* O157 from Dairy Cattle Associated with Two Cases of Haemolytic Uraemic Syndrome," Lancet. 2, p. 1043, 1986.

[81] Centers for Disease Control and Prevention, "Outbreak of *Escherichia coli* O157:H7 Infection Associated with Eating Fresh Cheese Curds-Wisconsin, June 1998," Morb. Mortal. Wkly. Rep. 49, pp. 911-913, 2000.

[82] G. Deschênes, C. Casenave, F. Grimont, J. C. Desenclos, S. Benoit, M. Collin, S. Baron, P. Marian, P. A. Grimont, and H. Nivet, "Cluster of Cases of Haemolytic Uraemic Syndrome Due to Unpasteurised Cheese," Pediatr. Nephrol. 10, pp. 203-205, 1996.

[83] C. Vernozy-Rozand, M. P. Montet, M. Berardin, C. Bavai, L. Beutin, "Isolation and Characterization of Shiga Toxin-Producing *Escherichia coli* Strains from Raw Milk Cheeses in France," Lett. Appl. Microbiol. 41, pp. 235-41, 2005.

[84] R. Stephan, S. Schumacher, S. Corti, G. Krause, J. Danuser, and L. Beutin, "Prevalence and Characteristics of Shiga Toxin-Producing *Escherichia coli* in Swiss Raw Milk Cheeses Collected at Producer Level," J. Dairy Sci. 91, pp. 2561-2565, 2008.

[85] D. Morgan, C. P. Newman, D. N. Hutchinson, A. M. Walker, B. Rowe, and F. Majid, "Verotoxin Producing *Escherichia coli* O157 Infections Associated with the Consumption of Yoghurt," Epidemiol. Infect. 111, pp. 181-187, 1993.

[86] C. Zweifel, N. Giezendanner, S. Corti, G. Krause, L. Beutin, J. Danuser, and R. Stephan, "Characteristics of Shiga Toxin-Producing *Escherichia coli* Isolated from Swiss Raw Milk Cheese within a 3-Year Monitoring Program," J. Food Prot. 73, pp. 88-91, 2010.

[87] M. Adams and Y. Motarjemi, "Basic Food Safety for Health Workers," World Health Organization, Geneva, 1999.

[88] P. Aureli, A. Costantini, L. Fenicia, M. Gianfranceschi, and L. Rainaldi, "Occurrence of Pathogenic *Escherichia coli* in Available Italian Soft Cheese," Arch. Lebensmittelhyg. 43, pp. 1-24, 1992.

[89] J. F. Frank and E. H. Marth, "Survey of Soft and Semisoft Cheese for Presence of Fecal Coliforms and Serotypes of Enteropathogenic *Escherichia coli*," J. Food Prot. 41, pp. 198-200, 1978.

[90] B. A. Glatz and S. A. Brudvig, "Survey of Commercially Available Cheese for Enterotoxigenic *Escherichia coli*." J. Food Prot. 43, pp. 395-398, 1980.

[91] Y. Watanabe, K. Ozasa, J. H. Mermin, P. M. Griffin, K. Masuda, S. Imashuku, and T. Sawada, "Factory Outbreak of *Escherichia coli* O157:H7 Infection in Japan," Emerg. Infect. Dis. 5, pp. 424-428, 1999.

[92] Y. Ozeki, T. Kurazono, A. Saito, T. Kishimoto, and M. Yamaguchi, "A Diffuse Outbreak of Enterohemorrhagic *Escherichia coli* O157:H7 Related to the Japanese-Style Pickles in Saitama, Japan," Kansenshogaku Zasshi. 77, pp. 493-498, 2003.

[93] A. M. Wendel, D. H. Johnson, U. Sharapov, J. Grant, J. R. Archer, T. Monson, C. Koschmann, and J. P. Davis, "Multistate Outbreak of *Escherichia coli* O157:H7 Infection Associated with Consumption of Packaged Spinach, August-September 2006: the Wisconsin Investigation," Clin. Infect. Dis. 48, pp. 1079-1086, 2009.

[94] A. Söderström, P. Osterberg, A. Lindqvist, B. Jönsson, A. Lindberg, S. B. Ulander, C. Welinder-Olsson, S. Löfdahl, B. Kaijser, B. De Jong, S. Kühlmann-Berenzon, S. Boqvist, E. Eriksson, E. Szanto, S. Andersson, G. Allestam, I. Hedenström, L. L. Muller, and Y. Andersson, "A Large *Escherichia coli* O157 Outbreak in Sweden Associated with Locally Produced Lettuce," Foodborne Pathog. Dis. 5, pp. 339-349, 2008.

[95] I. Friesema, G. Sigmundsdottir, K. van der Zwaluw, A. Heuvelink, B. Schimmer, C. de Jager, B. Rump, H. Briem, H. Hardardottir, A. Atladottir, E. Gudmundsdottir, and W. van Pelt, "An International Outbreak of Shiga Toxin-Producing *Escherichia coli* O157 Infection Due to Lettuce, September-October 2007," Euro Surveill. 13, p.19065, 2008.

[96] C. N. Berger, S. V. Sodha, R. K. Shaw, P. M. Griffin, D. Pink, P. Hand, and G. Frankel, "Fresh Fruit and Vegetables as Vehicles for the Transmission of Human Pathogens," Environ. Microbiol. 12, pp. 2385-2397, 2010.

[97] Cookson AL, Cooley WA, Woodward MJ, "The Role of Type 1 and Curli Fimbriae of Shiga Toxin-Producing *Escherichia coli* in Adherence to Abiotic Surfaces," Int. J. Med. Microbiol. 2002 Sep 292(3-4), pp. 195-205, 2002.

[98] X. Jiang, J. Morgan, and M. P. Doyle, "Fate of *Escherichia coli* O157:H7 in Manure-Amended Soil," Appl. Environ. Microbiol. 68, pp. 2605-2609, 2002.

[99] J. T. LeJeune, T. E. Besser, and D. D. Hancock, "Cattle Water Troughs as Reservoirs of *Escherichia coli* O157," Appl. Environ. Microbiol. 67, pp. 3053-3057, 2001.

[100] H. G. Yuk and D. L. Marshall, "Adaptation of *Escherichia coli* O157:H7 to pH Alters Membrane Lipid Composition, Verotoxin Secretion, and Resistance to Simulated Gastric Fluid Acid," Appl. Environ. Microbiol. 70, pp. 3500-3505, 2004.

[101] J. D. Oliver, "The Viable but Nonculturable State in Bacteria," J. Microbiol. 43, pp. 93-100, 2005.

[102] J. D. Oliver, "Recent Findings on the Viable but Nonculturable State in Pathogenic Bacteria," FEMS Microbiol. Rev.34, pp. 415-425, 2010.

[103] S. Makino, T. Kii, H. Asakura, T. Shirahata, T. Ikeda, K. Takeshi, and K. Itoh, "Does Enterohemorrhagic *Escherichia coli* O157:H7 Enter the Viable but Nonculturable State in Salted Salmon Roe?," Appl. Environ. Microbiol. 66, pp. 5536-5539, 2000.

[104] H. Asakura, K. Kawamoto, Y. Haishima, S. Igimi, S. Yamamoto, and S. Makino, "Differential Expression of the Outer Membrane Protein W (OmpW) Stress Response in Enterohemorrhagic *Escherichia coli* O157:H7 Corresponds to the Viable but Non-Culturable State," Res. Microbiol. 159, pp. 709-717, 2008.

[105] Y. Pei, J. Terajima, Y. Saito, R. Suzuki, N. Takai, H. Izumiya, T. Morita-Ishihara, M. Ohnishi, M. Miura, S. Iyoda, J. Mitobe, B. Wang, and H. Watanabe, "Molecular Characterization of Enterohemorrhagic *Escherichia coli* O157:H7 Isolates Dispersed Across Japan by Pulsed-Field Gel Electrophoresis and Multiple-Locus Variable-Number Tandem Repeat Analysis," Jpn. J. Infect. Dis. 61, pp. 58-64, 2008.

[106] H. Watanabe, J. Terajima, H. Izumiya, S. Iyoda, and K. Tamura, "PulseNet Japan: Surveillance System for the Early Detection of Diffuse Outbreak Based on the Molecular Epidemiological Method," Kansenshogaku Zasshi. 76, pp. 842-848 2002.

[107] NIID and MHLW, "Enterohemorrhagic *Escherichia coli* Infection in Japan as of May 2010," Infectious Agents Surveillance Report (IASR). 31, pp. 155-156, 2010.

[108] F. Kasuga, M. Hirota, M. Wada, T. Yunokawa, H. Toyofuku, M. Shibatsuji, H. Michino, T. Kuwasaki, S. Yamamoto, and S. Kumagai, "Archiving of Food Samples from Restaurants and Caterers - Quantitative Profiling of Outbreaks of Foodborne Salmonellosis in Japan," J. Food Prot. 67, pp. 2024-2032, 2004.
[109] http://www.codexalimentarius.net/web/index_en.jsp
[110] http://www.who.int/foodsafety/micro/jemra/en/index.html
[111] FAO/WHO, "Risk Assessment of Enterohaemorrhagic *Escherichia coli* (EHEC) in Meat and Meat Products: Inception Meeting, Dublin, Ireland," 4-8 September 2006.
[112] AFSSA, "Quantitative Risk Assessment for *Escherichia coli* O157:H7 in Frozen Beef Burgers Consumed at Home in France by Children under the Age of 16," 2007.
[113] RIVM, "RIVM Report 257851003- Risk Assessment of Shiga-Toxin Producing *Escherichia coli* O157 in Steak Tartare in the Netherlands," 2001.
[114] RIVM, "RIVM Report 284550008- Disease Burden in the Netherlands due to Infections with Shiga-Toxin Producing *Escherichia coli* O157," 2003.
[115] USDA/FSIS, "Risk Assessment of the Public Health Impact of *Escherichia coli* O157:H7 in Ground Beef," 2001.
[116] USDA/FSIS, "Comparative Risk Assessment for Intact (Non-Tenderized) and Non-Intact (Tenderized) Beef" 2002.
[117] P. Teunis, K. Takumi and K. Shinagawa, "Dose Response for Infection by *Escherichia coli* O157:H7 from Outbreak Data," Risk Analysis. 24, pp. 401-407, 2004.
[118] N. J. Strachan, M. P. Doyle, F. Kasuga, O. Rotariu, and I. D. Ogden, "Dose Response Modelling of *Escherichia coli* O157 Incorporating Data from Foodborne and Environmental Outbreaks," Int. J. Food Microbiol. 103, pp. 35-47, 2005.

Name:
Hiroshi Asakura

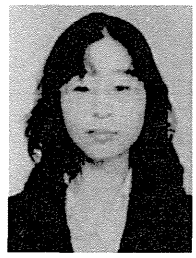
Affiliation:
Division of Biomedical Food Research, National Institute of Health Sciences

Address:
1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan

Brief Career:
2004- National Institute of Health Sciences
2007-2009 Postdoctoral Fellow, Max Plank Institute for Infection Biology, Germany

Selected Publications:
- "*Helicobacter pylori* HP0518 Affects Flagellin Glycosylation to Alter Bacterial Motility," Mol. Microbiol. Vol.78, pp. 1130-1144, 2010.
- "Gene Expression Profile of *Vibrio cholerae* in the Cold Stress-Induced Viable but Non-Culturable State," Environ Microbiol. Vol.9, pp. 869-879, 2007.
- "Viable but Nonculturable *Salmonella* Species Recovery and Systemic Infection in Morphine-Treated Mice," J. Infect Dis. Vol.186, pp. 1526-1529, 2002.

Academic Societies & Scientific Organizations:
- Japanese Society for Bacteriology (JSB)
- The Japan Society of Veterinary Science (JSVS)
- Japanese Society for Campylobacter Research (JSCR)



Name:
Yoshika Momose

Affiliation:
Division of Biomedical Food Research, National Institute of Health Sciences

Address:
1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan

Brief Career:
2006- Postdoctoral Fellow, the University of Tokyo
2010- National Institute of Health Sciences

Selected Publications:
- "Effect of Organic Acids on Inhibition of *Escherichia coli* O157:H7 Colonization in Gnotobiotic Mice Associated with Infant Intestinal Microbiota," Antonie van Leeuwenhoek, Vol.93, pp. 141-149, 2008.

Academic Societies & Scientific Organizations:
- Japanese Association of Germfree Life and Gnotobiology (JAGG)
- Japan Bifidus Foundation (JBF)
- Japanese Society for Bacteriology (JSB)

Name:
Fumiko Kasuga

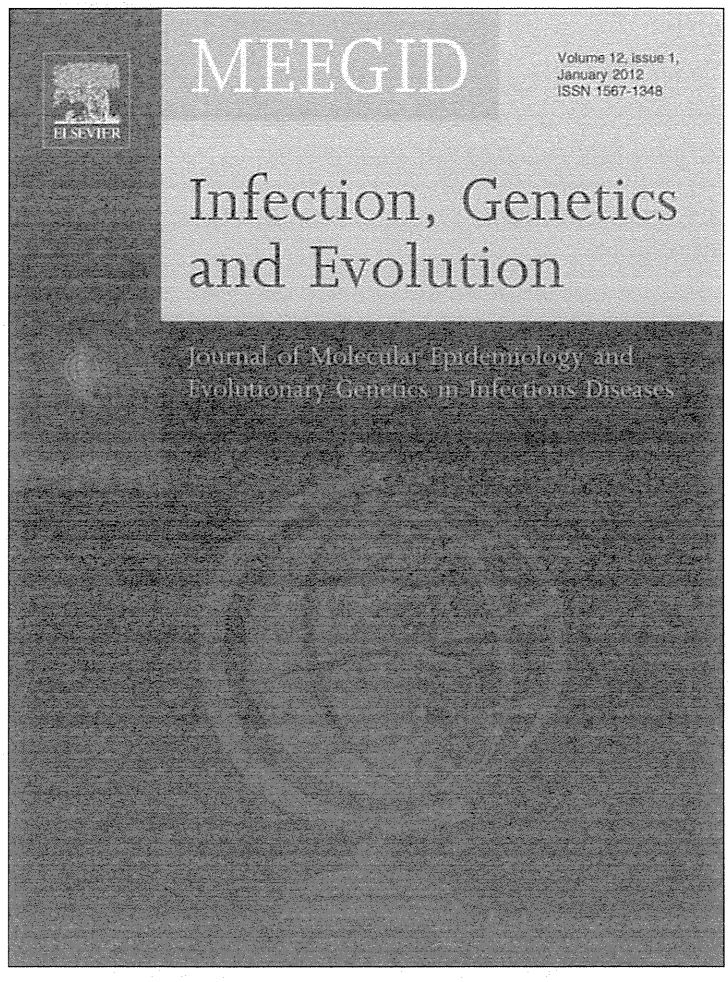
Affiliation:
Division of Biomedical Food Research, National Institute of Health Sciences

Address:
1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan

Brief Career:
1989 National Institute of Infectious Diseases
2002- National Institute of Health Sciences

Selected Publications:
- J. Iwahori et al., "Quantitative Risk Assessment of Vibrio Parahaemolyticus in Finfish: A Model of Raw Horse Mackerel Consumption in Japan," Risk Analysis, Vol.30, No.12, pp. 1817-1832, 2010.
- K. Kubota et al., "The Human Health Burden of Foodborne Infections Caused by *Campylobacter*, *Salmonella*, and *Vibrio parahaemolyticus* in Miyagi Prefecture, Japan," Foodborne Pathog Dis., Vol.5, No.5, pp. 641-8, 2008.

Academic Societies & Scientific Organizations:
- Society for Risk Analysis
- International Association of Food Protection
- International Commission of Microbiological Specifications for Foods



Infection, Genetics and Evolution 12 (2012) 94–101

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Intrahost passage alters SigB-dependent acid resistance and host cell-associated kinetics of *Listeria monocytogenes*

Hiroshi Asakura [a,*], Keiko Kawamoto [b], Yumiko Okada [a], Fumiko Kasuga [a], Sou-ichi Makino [b], Shigeki Yamamoto [a], Shizunobu Igimi [a]

[a] Division of Biomedical Food Research, National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan
[b] Research Center for Animal Hygiene and Food Safety, Obihiro University of Agriculture and Veterinary Medicine, Inada 2, Obihiro, Hokkaido 080-8555, Japan

ARTICLE INFO

Article history:
Received 5 May 2011
Received in revised form 13 October 2011
Accepted 15 October 2011
Available online 24 October 2011

Keywords:
Listeria monocytogenes
Acid resistance
SigB

ABSTRACT

Listeria monocytogenes is a foodborne pathogen that causes gastroenteritis, maternofetal infections and meningoencephalitis in humans. Here we report that an intrahost genome mutation alters bacterial acid resistance and the abilities for replication/invasion in tissue cell culture. Among the *L. monocytogenes* isolates from the recent outbreak in Japan, we found that one food strain, 668, exhibited the greatest acid resistance, whereas one human clinical strain, 690, sharing identical pulsed-field gel electrophoresis (PFGE) and ribotyping patterns, exhibited an acid-sensitive phenotype. Passage of the 668 food strain through the mouse intestine increased its acid sensitivity without altering the macrogenotypes, indicating intrahost alteration of the bacterial acid-resistant phenotype. Genetic and proteomic analyses revealed a link between acid resistance and SigB (RNA polymerase SigmaB subunit) activity. Compared with the strain 668, the clinical and 4 of 5 mice-passaged strains showed a mutation in the *rsbW* locus, whose product controls the regulation of SigB activity. Corresponding to the SigB activity, the host-passaged strains had reduced abilities to survive inside macrophages and to invade Caco-2 cells, compared with the food strain 668. Overall, we have demonstrated the first example of a host environment promoting the alteration of SigB-dependent acid resistance and host cell-associated actions of *L. monocytogenes*. Our study provides new insight into the potential role of intrahost environment in the process of bacterial evolution.

1. Introduction

Listeria monocytogenes is a Gram-positive, facultative intracellular bacterium, widely disseminated in foods and elsewhere in nature. Upon infection, this pathogen can cross the intestinal, placental and blood-brain barriers, leading to gastroenteritis, maternofetal infections and meningoencephalitis in humans (Lecuit, 2005). Mounting evidence suggests that this pathogen is resistant to various foreign stressors, including heat, cold, NaCl osmolarity, acidity, oxides, and ultraviolet (UV) radiation, which enables this bacterium to colonise dairy and meat products, the main causative agents of human listeriosis (Chaturongakul and Boor, 2006; Cotter and Hill, 2003; McLauchlin et al., 2004; Merrell and Camilli, 2002). Thus, improving our understanding of the molecular basis of this pathogen's stress-resistance system could lead to better control of transmission of this bacterium to humans.

The recent development of genome sequencing technology has revealed that the bacterial genome can evolve to adapt to *in vivo* conditions and may exhibit host imprints (Giraud et al., 2008; Price et al., 2010). For example, in uropathogenic *Escherichia coli*, the bacterial genome has evolved to alter the transcription of metabolic and virulence-associated genes for adaptation to the *in vivo* environment (Zdziarski et al., 2010). Such evidence has mainly been accumulated for the model organism *E. coli*, whereas little is known about how or even whether *L. monocytogenes* exhibits such genetic alterations as part of its evolution or adaptation. However, a recent study demonstrated that staphylococcal pathogenicity islands were transferred to the *L. monocytogenes* genome in bovine raw milk, implicating microbial evolution (Chen and Novick, 2009). This finding suggests possible *in vivo* mutational evolution of this pathogen because the intestinal environment includes a vast array of bacteriophage particles that can trigger such interbacterial genetic transfer (Grayson and Molineux, 2007).

We previously reported that *L. monocytogenes* isolates from infected individuals and cheeses showed high genetic similarity based on their pulsed-field gel electrophoresis (PFGE) and ribotype profiles, which provided a possible explanation for the first food-

Abbreviations: PFGE, pulsed-field gel electrophoresis; SNP, single nucleotide polymorphism; m.o.i, multiplicity of infection.

* Corresponding author. Tel.: +81 3 3700 1141x505; fax: +81 3 3700 9246.
E-mail address: hasakura@nihs.go.jp (H. Asakura).

1567-1348/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2011.10.014

borne listerial outbreak in Japan (Makino et al., 2005). As the causative foods were highly contaminated with this pathogen (Makino et al., 2005), it was thought that acid resistance should be a prerequisite for the survival of bacteria in the fermented products. In this study, we thus characterised the acid resistance of representative *L. monocytogenes* isolates from the outbreak. Our analysis revealed different levels of acid resistance in a food isolate and a clinical isolate (the food isolate was acid-resistant, and the clinical isolate was acid-sensitive), although the two isolates had identical macrogenotypes. We thus reasoned that the acid-resistant phenotype of *L. monocytogenes* might be altered during passage through the infected individual and subsequently initiated animal experiments to determine whether the host environment can alter acid resistance. We then attempted to elucidate the molecular basis of the intrahost alteration of this phenotype. Through genetic and proteomic analyses, we identified the candidate gene associated with the altered acid resistance in *L. monocytogenes*, which also affected the bacterial behaviours for cell invasion and intracellular replication.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth media

L. monocytogenes strains originating from a 2001 outbreak in Japan (Makino et al., 2005) were used in this study (Table 1). The bacteria were grown using routine methods on brain heart infusion (BHI) agar (Becton Dickinson) or in BHI broth at 37 °C unless otherwise indicated. These isolates were stocked at −80 °C immediately after the isolation, and from which the bacterial cultures were prepared independently for each testing.

2.2. Acid resistance test

To screen the acid resistance of representative *L. monocytogenes* strains from the outbreak (Table 1), bacterial cells grown in BHI broth at 37 °C for 20 h were washed twice with sterile phosphate-buffered saline (PBS, pH 7.4), and approximately 10^9 cells were inoculated into 10 ml of PBS supplemented with lactic acid (pH 4.0) (PBS-LA). After incubation for 24, 48 or 72 h, 100 µl of the suspension and several serial dilutions were plated onto BHI agar to determine viability counts. Separately, the survival of *L. monocytogenes* strains 668, 690 and mp1 in PBS-LA was further assessed in same manner except with different incubation times (0, 3, 6, 12, 24, and 48 h).

Table 1
Acid resistance of *L. monocytogenes* strains.

Strain	Origin	PFGE cluster*	Percentage survival in acidic conditions**		
			24 h	48 h	72 h
667	Cheese	A	2.14 ± 0.98E-04	ND***	ND
684	Clinical	A	2.86 ± 1.31E-04	ND	ND
673	Cheese	B	5.71 ± 2.64E-04	ND	ND
704	Clinical	B	2.86 ± 0.55E-04	ND	ND
669	Cheese	C	5.57 ± 1.36E-04	ND	ND
675	Cheese	C	7.86 ± 1.99E-05	ND	ND
685	Clinical	C	6.00 ± 2.95E-03	ND	ND
686	Clinical	C	2.14 ± 0.81E-04	ND	ND
670	Cheese	C	1.79 ± 1.03E-04	ND	ND
671	Cheese	C	2.21 ± 0.95E-04	ND	ND
676	Cheese	C	5.36 ± 3.48E-03	ND	ND
681	Cheese	C	2.50 ± 1.66E-03	ND	ND
668	Cheese	D	2.29 ± 1.44E-01	1.50 ± 1.46E-03	5.00 ± 3.90E-05
690	Clinical	D	4.50 ± 0.97E-04	ND	ND
677	Cheese	E	1.00E ± 0.86E-03	ND	ND
672	Cheese	F	1.43E ± 1.01E-04	ND	ND
689	Clinical	F	5.57E ± 2.52E-04	ND	ND

* PFGE clustering data were taken from Makino et al. (2005).
** The survival of bacteria in PBS containing lactic acid (pH 4.0) was examined. At the indicated time points, serial dilutions of bacterial suspensions were spread on BHI agar for CFU counting.
*** ND, Not detected, meaning that the viable numbers were less than 1.0×10^1 CFU/ml, equivalent to the percentage survival of approximately <1.0E-05.

2.3. Mouse passage of L. monocytogenes

Specific pathogen-free, female BALB/c mice ($n = 5$ per group, a total of 10 animals) were orally inoculated at 4–6 weeks of age with approximately $4.0–4.5 \times 10^8$ colony-forming units (CFUs) of *L. monocytogenes* strains 668 and 690. At 3 days post infection, the animals were sacrificed, and 0.5-g portions of the caecum contents were aseptically collected, homogenised in PBS and spread on PALCUM Listeria agar plates (MERCK) to recover *L. monocytogenes*. Fifty representative colonies of each strain were tested for survival after a 48-h incubation in PBS-LA, as described above. As a control, $1.2–1.7 \times 10^8$ cells of *L. monocytogenes* strains 668 and 690 were incubated in 10 ml of PBS for 72 h at 37 °C, and 50 colonies were collected from each suspension by plating on PALCUM Listeria agar plates. These colonies were also subjected to the acid sensitivity assay. Simultaneously, both strains were incubated at 37 °C for 3 days in BHI broth with subculture at 12-h intervals, and a representative 50 colonies collected by plating on PALCUM Listeria agar plates were tested for acid sensitivity as well. Each test was performed in triplicate sets independently.

2.4. Macrogenotyping (PFGE and ribotyping) analyses

The PFGE analysis was performed using the CHEFF MAPPER system (Bio-Rad) with either *Apa*I or *Asc*I, as described by Makino et al. (2005). Ribotyping analysis was performed using the RiboPrinter microbial characterisation system (DuPont, Wilmington, DE) according to the manufacturer's instructions.

2.5. Two-dimensional gel electrophoresis (2-DE) profiling and protein identification

Approximately 4.0×10^9 cells of *L. monocytogenes* strains 668 and 690 grown in BHI broth at 37 °C for 20 h were incubated in 50 ml of PBS-LA for 6 h at 37 °C. The bacterial cells were then collected, resuspended in 20 mM Tris–HCl (pH 7.5) and homogenised by intermittent sonication to extract proteins, as described by Asakura et al. (2007a). After electrophoresis on IPG strips (11 cm, pH 4–7, Bio-Rad), the samples were equilibrated, loaded onto 12% (w/v) acrylamide gels, and stained with Coomassie Brilliant Blue. Comparative quantitative analysis of the spot intensities was performed using PDQuest (Bio-Rad), and spots showing more than a 5-fold difference in intensity between the samples were excised and in-gel digested with trypsin (Pierce), as described (Asakura et al., 2007a). Peptide identification was performed by PMF analysis using an ABI4800 (LifeScience Technology), as described (Asakura et al., 2007a).

2.6. Semi-quantitative RT-PCR

Total RNA was isolated, and 1 µg of which was subjected to synthesise cDNA as described by Asakura et al. (2007b). For sample preparation, the bacteria were incubated in PBS-LA for 0, 30, 60, or 120 min. One microgram of each cDNA sample was then subjected to PCR using a series of oligonucleotide primers (Table S1) under the following conditions: 94 °C for 5 min (1 cycle); 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s (30 cycles); and 72 °C for

7 min (1 cycle). The PCR products were visualised with ethidium bromide on 2% agarose gels.

2.7. Western blot

Bacterial lysates were prepared in Laemmli buffer (60 mM Tris (pH 6.8), 2% (w/v) SDS, 10% (w/v) glycerol, 0.1% (w/v) bromophenol blue) and loaded onto 12% (w/v) acrylamide gels. The proteins on the gel were transferred onto PVDF membranes (Millipore) and incubated with blocking buffer (0.05 M Tris (pH 7.4), 0.2 M NaCl, 0.1% (w/v) Tween, 3% (w/v) BSA) for 1 h at room temperature. We used a primary antiserum against *L. monocytogenes* SigB (produced in-house) in the blocking buffer. An HRP-labelled goat anti-rabbit IgG antibody (GE Healthcare Bioscience) was used as the secondary antibody. The signals were chemiluminescently detected with the ECL detection system (GE Healthcare Bioscience) according to the manufacturer's instructions.

2.8. Genome sequencing

Genomic DNA was extracted from *L. monocytogenes* strains 668 and 690 with the DNeasy Tissue kit (Qiagen). Whole-genome shotgun sequencing was performed on the Illumina Genome Analyser (GA) II standard platform as per the manufacturer's recommendations, the data of which were deposited into DNA Databank of Japan (DDBJ) under the Accession No. DRA000377. Mapping was performed with Velvet software (Zerbino and Birney, 2008) and the contig sequences were used to detect SNPs between the two genome samples, generated by using MUMmer 3.0 according to the documentation at the website (http://mummer.sourceforge.net/).

2.9. Nucleotide sequencing of sigB operon in L. monocytogenes

Nucleotide sequences of approximately 4.7 kb-*sigB* operon (*rsbR–rsbS–rsbT–rsbU–rsbV–rsbW–sigB*) were extracted from the mapping data of strains 668 and 690, which were then completed by the partial compensation with DNA sequencing in ABI3730x DNA analyser according to the manufacturer's instructions. The obtained sequences were aligned, and the nucleotide sequences of *rsbW* ORF were further analysed for the strains mp1–5. These sequences were deposited on DNA Databank of Japan (DDBJ) as Accession Nos. AB665219 (strain 668), AB665220 (strain 690), AB665221 (mp1–3) and AB665222 (mp5).

2.10. Intramacrophage survival and Caco-2 invasion assay

J774 murine macrophages (3.0×10^5 cells) were seeded in RPMI1640 (Gibco) in 24-well tissue culture plates (TPPs) and incubated in a humidified CO_2 incubator at 37 °C for 24 h prior to use. The cells were infected with *L. monocytogenes* for 15 min at a multiplicity of infection (m.o.i.) of 20. The cells were then washed three times with PBS to eliminate extracellular bacteria and supplied with fresh medium containing gentamicin (30 μg ml^{-1}, Sigma) to kill any remaining extracellular bacteria. At 0, 2, or 4 h post infection, the cells were gently washed four times with PBS and lysed with 0.1% (w/v) Triton X-100 (Sigma) for 20 min. The intracellular bacteria were then enumerated by plating the suspensions onto BHI agar plates. The Caco-2 invasion assay was performed in a similar manner, except with a different infection time period (1 h) and a series of m.o.i. (20, 40 and 80).

2.11. Mice infection assay

Female BALB/c mice aged 5 weeks ($n = 10$) were infected intravenously with 5×10^4 of *L. monocytogenes* strains 668, 690, and mp1 that were grown in BHI broth at 37 °C for 20 h. Mortality was scored once a day for 14 days post inoculation.

2.12. Statistical analysis

Data for acid resistance and cell survival/invasion assays are represented as the means ± standard deviation (SD) obtained from three independent experiments. The *in vivo/vitro* passage assays are performed in three independent experiments. Statistical significance was calculated using Student's *t*-test ($p < 0.01$).

3. Results

3.1. Epidemiological background suggests intrahost alteration of acid resistance in L. monocytogenes

In the *L. monocytogenes* outbreak that occurred in Japan in 2001, cheese products were identified as the causative agent (Makino et al., 2005). Because these cheeses had been fermented by lactic acid bacteria, it was thought that acid resistance might be one of the bacterial determinants for survival in this type of habitat. Rep-

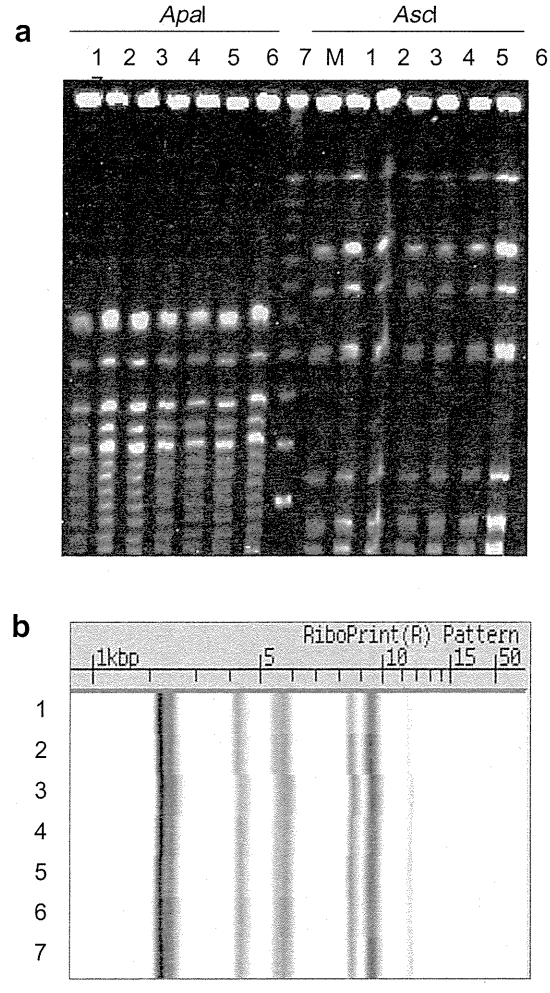


Fig. 1. Macromolecular typing of *L. monocytogenes* isolates. (A) Pulsed-field gel electrophoresis (PFGE) of *L. monocytogenes* strains. The acid-resistant food isolate, 668 (lane 1), the acid-sensitive human clinical isolate, 690 (lane 2), and the mouse-passaged variants of strain 668 that exhibited acid-sensitive phenotypes (mp1–5) (lanes 3–7) were subjected to PFGE with *ApaI* (left) or *AscI* (right). M represents the lambda ladder marker (48.5–1000 kb). (B) Riboprinting analysis of the *L. monocytogenes* isolates. Sample name and lane numbers correspond to those in panel A.