5. **Cohen, J.** 2002. Public health. Looking for vaccines that pack a wallop without the side effects. Science **298**:2314.
6. **Di Giulio, D. B., and P. B. Eckburg.** 2004. Human monkeypox: an emerging zoonosis. Lancet Infect. Dis. **4**:15–25.
7. **Esposito, J. J., and F. Fenner.** 2001. Poxviruses. p. 2887–2921. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, Pa.
8. **Galmiche, M. C., J. Goenaga, R. Wittek, and L. Rindisbacher.** 1999. Neutralizing and protective antibodies directed against vaccinia virus envelope antigens. Virology **254**:71–80.
9. **Guarner, J., B. J. Johnson, C. D. Paddock, W. J. Shieh, C. S. Goldsmith, M. G. Reynolds, I. K. Damon, R. L. Regnery, and S. R. Zaki.** 2004. Monkeypox transmission and pathogenesis in prairie dogs. Emerg. Infect. Dis. **10**:426–431.
10. **Halsell, J. S., J. R. Riddle, J. E. Atwood, P. Gardner, R. Shope, G. A. Poland, G. C. Gray, S. Ostroff, R. E. Eckart, D. R. Hospenthal, R. L. Gibson, J. D. Grabenstein, M. K. Arness, and D. N. Tornberg.** 2003. Myopericarditis following smallpox vaccination among vaccinia-naïve US military personnel. JAMA **289**:3283–3289.
11. **Hashizume, S., H. Yoshizawa, M. Morita, and K. Suzuki.** 1985. Properties of attenuated mutant of vaccinia virus, LC16m8, derived from Lister strain, p. 88–99. *In* G. V. Quinnan (ed.), Vaccinia viruses as vectors for vaccine antigens. Elsevier, Amsterdam, The Netherlands.
12. **Hatakeyama, S., K. Moriya, M. Saijo, Y. Morisawa, I. Kurane, K. Koike, S. Kimura, and S. Morikawa.** 2005. Persisting humoral antiviral immunity within the Japanese population after the discontinuation in 1976 of routine smallpox vaccinations. Clin. Diagn. Lab. Immunol. **12**:520–524.
13. **Hollinshead, M., G. Rodger, H. Van Eijl, M. Law, R. Hollinshead, D. J. Vaux, and G. L. Smith.** 2001. Vaccinia virus utilizes microtubules for movement to the cell surface. J. Cell Biol. **154**:389–402.
14. **Hooper, J. W., D. M. Custer, and E. Thompson.** 2003. Four-gene-combination DNA vaccine protects mice against a lethal vaccinia virus challenge and elicits appropriate antibody responses in nonhuman primates. Virology **306**: 181–195.
15. **Hooper, J. W., E. Thompson, C. Wilhelmsen, M. Zimmerman, M. A. Ichou, S. E. Steffen, C. S. Schmaljohn, A. L. Schmaljohn, and P. B. Jahrling.** 2004. Smallpox DNA vaccine protects nonhuman primates against lethal monkeypox. J. Virol. **78**:4433–4443.
16. **Hu, G., M. J. Wang, M. J. Miller, G. N. Holland, D. A. Bruckner, R. Civen, L. A. Bornstein, L. Mascola, M. A. Lovett, B. J. Mondino, and D. A. Pegues.** 2004. Ocular vaccinia following exposure to a smallpox vaccinee. Am. J. Ophthalmol. **137**:554–556.
17. **Katz, E., B. M. Ward, A. S. Weisberg, and B. Moss.** 2003. Mutations in the vaccinia virus A33R and B5R envelope proteins that enhance release of extracellular virions and eliminate formation of actin-containing microvilli without preventing tyrosine phosphorylation of the A36R protein. J. Virol. **77**:12266–12275.
18. **Khodakevich, L., Z. Jezek, and D. Messinger.** 1988. Monkeypox virus: ecology and public health significance. Bull. W. H. O. **66**:747–752.
19. **Kidokoro, M., M. Tashiro, and H. Shida.** 2005. Genetically stable and fully effective smallpox vaccine strain constructed from highly attenuated vaccinia LC16m8. Proc. Natl. Acad. Sci. USA **102**:4152–4157.
20. **Kimura, M., and H. Sakai.** 1996. Complications of smallpox vaccination. Clin. Virol. **24**:30–39.
21. **Kitamura, T.** 1999. Smallpox eradication and future prospect of smallpox vaccine. Clin. Virol. **27**:378–384.
22. **Lane, J. M., and J. Goldstein.** 2003. Adverse events occurring after smallpox vaccination. Semin. Pediatr. Infect. Dis. **14**:189–195.
23. **Miller, J. R., N. M. Cirino, and E. F. Philbin.** 2003. Generalized vaccinia 2 days after smallpox revaccination. Emerg. Infect. Dis. **9**:1649–1650.
24. **Morikawa, S., T. Sakiyama, H. Hasegawa, M. Saijo, A. Maeda, I. Kurane, G. Maeno, J. Kimura, C. Hirama, T. Yoshida, Y. Asahi-Ozaki, T. Sata, T. Kurata, and A. Kojima.** 2005. An attenuated LC16m8 smallpox vaccine: analysis of full-genome sequence and induction of immune protection. J. Virol. **79**:11873–11891.
25. **Murphy, J. G., R. S. Wright, G. K. Bruce, L. M. Baddour, M. A. Farrell, W. D. Edwards, H. Kita, and L. T. Cooper.** 2003. Eosinophilic-lymphocytic myocarditis after smallpox vaccination. Lancet **362**:1378–1380.
26. **Nagata, N., T. Iwasaki, Y. Ami, A. Harashima, I. Hatano, Y. Suzaki, K. Yoshii, T. Yoshii, A. Nomoto, and T. Kurata.** 2001. Comparison of neuropathogenicity of poliovirus type 3 in transgenic mice bearing the poliovirus receptor gene and cynomolgus monkeys. Vaccine **19**:3201–3208.
27. **Nagata, N., H. Shimizu, Y. Ami, Y. Tano, A. Harashima, Y. Suzaki, Y. Sato, T. Miyamura, T. Sata, and T. Iwasaki.** 2002. Pyramidal and extrapyramidal involvement in experimental infection of cynomolgus monkeys with enterovirus 71. J. Med. Virol. **67**:207–216.
28. **Neubauer, H., U. Reischl, S. Ropp, J. J. Esposito, H. Wolf, and H. Meyer.** 1998. Specific detection of monkeypox virus by polymerase chain reaction. J. Virol. Methods **74**:201–207.
29. **Newsome, T. P., N. Scaplehorn, and M. Way.** 2004. SRC mediates a switch from microtubule- to actin-based motility of vaccinia virus. Science **306**:124–129.
30. **Reed, K. D., J. W. Melski, M. B. Graham, R. L. Regnery, M. J. Sotir, M. V. Wegner, J. J. Kazmierczak, E. J. Stratman, Y. Li, J. A. Fairley, G. R. Swain, V. A. Olson, E. K. Sargent, S. C. Kehl, M. A. Frace, R. Kline, S. L. Foldy, J. P. Davis, and I. K. Damon.** 2004. The detection of monkeypox in humans in the Western Hemisphere. N. Engl. J. Med. **350**:342–350.
31. **Saurina, G., S. Shirazi, J. M. Lane, B. Daniel, and L. DiEugenia.** 2003. Myocarditis after smallpox vaccination: a case report. Clin. Infect. Dis. **37**: 145–146.
32. **Schmelz, M., B. Sodeik, M. Ericsson, E. J. Wolffe, H. Shida, G. Hiller, and G. Griffiths.** 1994. Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. J. Virol. **68**:130–147.
33. **Semba, R. D.** 2003. The ocular complications of smallpox and smallpox immunization. Arch. Ophthalmol. **121**:715–719.
34. **Smith, G. L., A. Vanderplasschen, and M. Law.** 2002. The formation and function of extracellular enveloped vaccinia virus. J. Gen. Virol. **83**:2915–2931.
35. **Takahashi-Nishimaki, F., S. Funahashi, K. Miki, S. Hashizume, and M. Sugimoto.** 1991. Regulation of plaque size and host range by a vaccinia virus gene related to complement system proteins. Virology **181**:158–164.
36. **Takahashi-Nishimaki, F., K. Suzuki, M. Morita, T. Maruyama, K. Miki, S. Hashizume, and M. Sugimoto.** 1987. Genetic analysis of vaccinia virus Lister strain and its attenuated mutant LC16m8: production of intermediate variants by homologous recombination. J. Gen. Virol. **68**:2705–2710.
37. **Weltzin, R., J. Liu, K. V. Pugachev, G. A. Myers, B. Coughlin, P. S. Blum, R. Nichols, C. Johnson, J. Cruz, J. S. Kennedy, F. A. Ennis, and T. P. Monath.** 2003. Clonal vaccinia virus grown in cell culture as a new smallpox vaccine. Nat. Med. **9**:1125–1130.
38. **Whitman, T. J., M. A. Ferguson, and C. F. Decker.** 2003. Cardiac dysrhythmia following smallpox vaccination. Clin. Infect. Dis. **37**:1579–1580.
39. **Zaucha, G. M., P. B. Jahrling, T. W. Geisbert, J. R. Swearengen, and L. Hensley.** 2001. The pathology of experimental aerosolized monkeypox virus infection in cynomolgus monkeys (*Macaca fascicularis*). Lab. Investig. **81**: 1581–1600.

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Laboratory Diagnostic Systems for Ebola and Marburg Hemorrhagic Fevers Developed with Recombinant Proteins

Masayuki Saijo,[1]* Masahiro Niikura,[1]† Tetsuro Ikegami,[1]‡ Ichiro Kurane,[1] Takeshi Kurata,[2] and Shigeru Morikawa[1]

Department of Virology 1, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan,[1] and Department of Pathology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-6840, Japan[2]

Ebola virus and *Marburg virus* (EBOV and MARV, respectively) of the family *Filoviridae* cause hemorrhagic fever with high mortality rates, sometimes reaching 50 to 90% of infected individuals, in humans and nonhuman primates (10, 16, 47). EBOV consists of four species, Zaire EBOV, Sudan EBOV, Ivory Coast EBOV, and Reston EBOV, which were first isolated in the Democratic Republic of Congo, Sudan, Ivory Coast, and the Philippines, respectively (47). MARV consists of one species, Lake Victoria MARV. Public health concerns about filovirus infection have increased in recent years. First, there have recently been large outbreaks of hemorrhagic fevers caused by EBOV (Ebola hemorrhagic fever [EHF]) and MARV (Marburg hemorrhagic fever [MHF]) in Africa; EHF outbreaks occurred in the Democratic Republic of Congo and Uganda in 1995 and 2000, respectively, and MHF outbreaks occurred in the Democratic Republic of Congo in 1998–1999 and in Angola in 2004–2005 (1, 7, 27, 56, 58–60). Second, there is a possibility that filoviruses may be used as bioweapons. In this regard, filoviruses are classified as category A warfare agents by the U.S. government and are considered to pose a great risk to international security, along with anthrax, botulism, tularemia, and smallpox (2).

As the magnitude of international trade and travel is continuously increasing, there is a significant risk that the hemorrhagic fever viruses could be introduced to virus-free countries from areas where they are endemic. Therefore, the development of laboratory diagnostic systems for EHF and MHF is an important subject even in countries without viral hemorrhagic fevers. Manipulation of infectious hemorrhagic fever viruses such as EBOV, MARV, Crimean-Congo hemorrhagic fever virus, and Lassa virus requires a biosafety level 4 (BSL-4) laboratory, which is designed for work with dangerous and exotic agents that pose a high risk of laboratory infection and life-threatening disease. However, BSL-4 laboratories are only available in a limited group of countries, such as the United States, Canada, France, the United Kingdom, Germany, South Africa, Sweden, and Russia.

To get around the need for BSL-4 laboratories, recombinant viral antigens are used for immunodiagnostics. The recombinant proteins of these viruses have been expressed, and serological diagnostic methods have been developed using the recombinant proteins. Antigen detection systems have also been developed using recombinant antigens. In this article, recent progress in the development of diagnostic methods for EHF and MHF is reviewed.

EBOLA AND MARBURG HEMORRHAGIC FEVERS

Structure of EBOV and MARV virions. Electron microscopic examination revealed that EBOV and MARV virions are pleomorphic, appearing as either long filamentous forms or in shorter U-shaped, 6-shaped, or circular configurations. The filamentous forms vary greatly in length (up to 14,000 nm), with mean unit lengths of virions of about 1,200 and 860 nm for EBOV and MARV, respectively (47). The virus genome of EBOV is almost 19 kb long and encodes seven viral proteins, namely, nucleoprotein (NP), polymerase cofactor (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), matrix protein (VP24), and RNA-dependent RNA polymerase (L), with an additional soluble glycoprotein (sGP) produced from an edited GP mRNA. The genes are arranged in the order 3′-NP-VP35-VP40-GP-VP30-VP24-L-5′. The virus genome of MARV has similar characteristics to EBOV, except for the expression of the soluble glycoprotein produced from edited GP mRNA (47). The nucleocapsid complexes of filoviruses consist of the nonsegmented negative-strand RNA genome, NP, polymerase L, VP35, and VP30. The structural proteins, VP40 and VP24, represent viral matrix proteins connecting the nucleocapsid to the viral envelope. The envelope GP is an integral membrane protein which forms spike-like protrusions on the surface of the virion (11, 13). GP mediates virus entry into susceptible cells through receptor binding and plays an important role in inducing neutralizing antibodies.

Diseases caused by EBOV and MARV. Filovirus infections, in general, are the most severe of the viral hemorrhagic fevers. Humans are usually infected with EBOV or MARV through close contact with the contaminated blood, tissues, and/or excretions of viremic animals, including patients with filovirus infections. After an incubation period of 4 to 10 days, infected individuals abruptly develop flu-like symptoms characterized by fever, chills, malaise, and myalgia. Subsequently, patients usually develop the signs and symptoms that indicate systemic involvement, such as prostration and gastrointestinal (anorexia, nausea, vomiting, abdominal pain, and diarrhea), respiratory (chest pain, shortness of breath, and cough), vascular (conjunctival injection, postural hypotension, and edema), and neurological (headache, confusion, and coma) manifestations.

* Corresponding author. Mailing address: Special Pathogens Laboratory, Department of Virology 1, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771, ext. 320. Fax: 81-42-561-2039. E-mail: msaijo@nih.go.jp.

† Present address: Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824.

‡ Present address: Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555-1019.

TABLE 1. Outbreaks of EHF and MHF

Virus	Country(ies)	Year(s)	No. of deaths/no. of patients	Description
Lake Victoria MARV	Germany and Serbia/Herzegovina	1967	7/31	Origin of MARV responsible for the outbreak was a monkey imported from Uganda.
	Zimbabwe and South Africa	1975	1/3	Index case was infected with MARV in Zimbabwe; nosocomial infection occurred in a hospital in South Africa.
	Kenya	1980	1/2	
	Kenya	1987	1/1	
	Democratic Republic of Congo	1998–1999	52/76	Prospective study on MHF indicated that there were approximately 150 patients with MHF in this outbreak.
	Angola	2004–2005	357/423 (as of 7 July 2005)	Mortality rate exceeded 80%; many children suffered from MHF in this outbreak.
Sudan EBOV	Sudan	1976	151/284	First documented outbreak of VHF due to Sudan EBOV.
Zaire EBOV	Democratic Republic of Congo	1976	280/318	First documented outbreak of VHF due to Zaire EBOV.
Zaire EBOV	Democratic Republic of Congo	1977	1/1	Sporadic outbreak.
Sudan EBOV	Sudan	1979	22/34	
Reston EBOV	United States	1989–1990	0/4	Outbreak of EHF in nonhuman primates imported from the Philippines. Four persons were confirmed to be infected with Reston EBOV without any symptoms.
Reston EBOV	Italy	1992	0/0	Outbreak of EHF in nonhuman primates imported from the Philippines.
Ivory Coast EBOV	Ivory Coast	1994	0/1	A veterinarian was infected when she handled a dead chimpanzee.
Zaire EBOV	Democratic Republic of Congo	1995	244/315	The epicenter of this outbreak is Kikwit, Democratic Republic of Congo.
Zaire EBOV	Gabon	1996	21/31	
Zaire EBOV	Gabon and South Africa	1996	45/60	Nosocomial infection occurred in a hospital in which a nurse who took care of a doctor that was transferred from Gabon died.
Sudan EBOV	Uganda	2000	149/394	The epicenter of this EHF outbreak was the Gulu District in Uganda.
Zaire EBOV	Gabon and the Democratic Republic of Congo	2001–2002	69/92	The outbreak area was close to the border between the two countries.
Zaire EBOV	Democratic Republic of Congo	2003	29/35	Thirteen of the cases were laboratory confirmed and 130 were epidemiologically linked.
Sudan EBOV	Sudan	2004	7/17	
Zaire EBOV	Democratic Republic of Congo	2005	9/12 (as of 16 June 2005)	One case was virologically confirmed (http://www.who.int/csr/don/2005_06_16/en/index.html).

Bleeding is manifested as petechiae, ecchymosis, uncontrolled oozing from venipuncture sites and gingiva, mucosal hemorrhages, and bloody diarrhea. In later stages, the general condition of patients deteriorates due to multiorgan failure, including disseminated intravascular coagulopathy, resulting in death (4, 14, 42, 47).

Epidemiology of Ebola and Marburg hemorrhagic fevers. The outbreaks caused by EBOV and MARV are summarized in Table 1. The first documented MHF outbreak occurred in Germany and then in Yugoslavia in 1967 (36). Technicians and scientists suffered from MHF after they manipulated tissue materials collected from African green monkeys imported from Uganda. It was suggested that the monkeys had already been infected with MARV when imported. Three sporadic cases of MHF were reported in Zimbabwe (1975) and Kenya (1980 and 1987) (8, 12, 24, 49). From 1998 to 1999, there was a large outbreak in the Democratic Republic of Congo (1). The largest outbreak of MHF occurred in Uige Province, Angola, in 2004, and 374 patients have been reported, with a mortality rate of over 88%, as of 24 August 2005 (http://www.who.int/csr/don/2005_08_24/en/index.html).

The first recognized outbreaks of EHF occurred in the Democratic Republic of Congo, formerly Zaire, and Sudan in 1976 (3, 26, 54, 55). After the discovery of EBOV in 1976, several African countries were struck by outbreaks of EHF caused by one of the three known human-pathogenic EBOV species (Zaire EBOV, Sudan EBOV, and Ivory Coast EBOV) (12, 17, 27, 31, 52, 57–59, 61, 62). Outbreaks of EHF caused by the other EBOV strain, Reston EBOV, occurred among cynomolgus macaques imported from the Philippines to the United States in 1989 (23). Reston EBOV was also introduced to the United States in 1989, 1990, and 1996, and to Italy in 1992, through importation of infected monkeys from the Philippines (5, 6, 23, 63). It is noteworthy that the incidences of EHF and MHF outbreaks are increasing and that the number of patients suffering from EHF or MHF is on the rise (Table 1).

Several cases of nosocomial infections of EBOV and MARV outside the areas of endemicity have been reported. A health-

care worker who took care of severely ill patients transported from Zimbabwe acquired MARV infection (15), and a healthcare worker was infected with Zaire EBOV after taking care of a severely ill patient transported from Gabon (41). The severely ill patient was a medical doctor who was infected with Zaire EBOV from an EHF patient in Gabon. Both nosocomial infections occurred in South Africa.

CURRENT LABORATORY DIAGNOSTICS FOR EHF AND MHF

Overview of current laboratory diagnostics for EHF and MHF. In outbreaks of EHF and MHF, infections are confirmed by various laboratory diagnostic methods. These include virus isolation, reverse transcription-PCR (RT-PCR), including real-time quantitative RT-PCR, antigen-capture enzyme-linked immunosorbent assay (ELISA), antigen detection by immunostaining, and IgG- and IgM-ELISA using authentic virus antigens (9, 18, 28–30, 32, 48, 50, 53, 64). Histological techniques, including antigen detection by immunohistochemical analyses, are sensitive methods, particularly for postmortem diagnosis (64). Diagnosis by detection of virus antigens is suitable for patients in the early stage of illness, while serological diagnosis by the detection of specific IgM and IgG antibodies is suitable for patients in a relatively late stage of illness. The former is especially suitable for patients who die before an antibody response is mounted. Diagnostics for viral hemorrhagic fevers, including EHF and MHF, must be sensitive, specific, and reliable because misdiagnosis of viral hemorrhagic fevers may bring huge turmoil to society. Therefore, the diagnosis of EHF and MHF must not rely on any single diagnostic method alone. The risk of misdiagnosis must be extremely minimized. In actual EHF or MHF outbreak areas, patients with EHF or MHF must be isolated. This indicates that a false-positive result will put an individual at unnecessary risk of infection by making the person be placed in a high-risk environment such as an isolation ward. A false-negative result will allow persons who are infected with EBOV or MARV to be released into the community with the understanding that they do not have viral hemorrhagic fever, when in fact they have the potential to become highly contagious and cause person-to-person transmission of these viruses in the community. In Africa, Lassa fever and Crimean-Congo hemorrhagic fever are also endemic. Therefore, the diagnosis of viral hemorrhagic fevers must rely on multiple diagnostic assays for viral hemorrhagic fevers in a comprehensive manner.

Virus isolation. Virus isolation is a basic, simple, and sensitive method for diagnosis of EHF and MHF. EBOV and MARV grow well in a large variety of cell lines, but Vero cells and Vero E6 cells are commonly used. Specimens such as blood must be sent to BSL-4 laboratories, which are located in developed countries, from an outbreak area, which is often very remote. The shipment of specimens for virus isolation under favorable conditions (cold chain during the period from shipment to arrival) is often difficult. Therefore, diagnostic criteria based on virus isolation alone will not yield an etiologic diagnosis. A panel of monoclonal antibodies to the recombinant NPs (rNPs) of Zaire EBOV, Reston EBOV, and Lake Victoria MARV was established by our group (20, 39, 40, 44). Monoclonal antibodies specific only for NP of Zaire EBOV, for NP of Reston EBOV, for NPs of Zaire and Sudan EBOV, for NPs of Zaire and Reston EBOV, and for NP of Lake Victoria MARV alone were developed. By using these monoclonal antibodies, it became possible to serologically identify the species of EBOV and MARV isolates.

RT-PCR, including real-time quantitative RT-PCR. Molecular diagnostic methods for EHF and MHF by RT-PCR were developed and evaluated well in the epidemic setting, especially in the EHF outbreaks in the Democratic Republic of Congo in 1995, in Gabon in 1996, and in Uganda in 2000 (32, 48, 50). During the EHF outbreak in Uganda in 2000, a nested RT-PCR using primer sets designed specifically for the NP region of the Sudan EBOV Gulu strain, which was the causative EBOV for the outbreak, was developed (50). Recently, real-time quantitative RT-PCR methods for EHF and MHF were also developed (9, 18, 50, 53). The real-time quantitative RT-PCR method developed by Drosten et al. is a one-step RT-PCR with the addition of the DNA-intercalating dye SYBR green I using the primer set Filo-A (5′-ATCGGAATT TTTCTTTCTCATT-3′) and Filo-B (5′-ATGTGGTGGGTTA TAATAATCACTGACATG-3′), which was designed for the amplification of L genes from both EBOV and MARV (Table 2) (9, 48). On the other hand, the real-time quantitative RT-PCR methods developed by other investigators are based on the technology of TaqMan probe-based quantitative RT-PCR, using a designated primer set with a fluorogenic TaqMan probe that is labeled at the 5′ end with the reporter dye 6-carboxyfluorescein and at the 3′ end with a quencher tag (18, 50, 53). The primer sequences used for RT-PCR, nested RT-PCR, and real-time quantitative RT-PCR are summarized in Table 2. These molecular diagnostics for EHF and MHF have been proven to be sensitive, specific, and efficacious in the diagnosis of filovirus infections. Although RT-PCR assays, especially nested RT-PCR and real-time quantitative RT-PCR, are useful, false-positive and false-negative results must always be excluded. The sensitivities of the RT-PCR systems used in several laboratories vary (38).

Antigen detection ELISAs for EHF and MHF. When infection with EBOV or MARV becomes fatal, patients usually die before the antibody response. This fact suggests that serological diagnostics are suitable for the diagnosis of infection in patients who survive but not in those who succumb to the infection. High titers of infectious filovirus are present in the blood and tissues of patients at the early stage of illness, suggesting that the detection of virus antigens is suitable for diagnosis of EHF and MHF at an early stage. Antigen-capture ELISA was developed for the detection of EBOV antigens; it was used in clinical settings such as EHF outbreaks in the Democratic Republic of Congo in 1995, in Gabon in 1996, and in Uganda in 2000 and was confirmed to be efficacious in diagnosis of EHF (28–30, 50). In the antigen-capture ELISA, a pool of monoclonal antibodies to the Zaire and Sudan EBOVs and rabbit sera raised to the Zaire and Sudan EBOVs were used as capture and detection antibodies, respectively (28, 29). Antigen-capture ELISA systems to detect EBOV and MARV antigens have also been developed by other groups, including ours (20, 34, 35, 39, 44). The target proteins are NP, VP40, and GP. The characteristics of the developed EBOV- and MARV-specific antigen-capture ELISAs are summarized in Table 3. Saijo et al. developed filovirus antigen detection

TABLE 2. Primers used for RT-PCR amplification of EBOV sequences and targeted genes, with predicted sizes of amplified DNA products

Assay system	Primer or probe[a]	Sequence (sense) (5′–3′)	Target gene (size of amplicon [bp])	Reference
RT-PCR	EBO-GP1 (F)	AATGGGCTGAAAATTGCTACAATC	EBOV GP (579)	48
	EBO-GP2 (R)	TTTTTTTAGTTTCCCAGAAGGCCCACT		
RT-PCR	FILO-A (F)	ATCGGAATTTTTCTTTCTCATT	Filovirus L (419)	48
	FILO-B (R)	ATGTGGTGGGTTATAATAATCACTGACATG		
RT-PCR	RES-NP1	GTATTTGGAAGGTCATGGATTC	Reston EBOV NP (337)	48
	RES-NP2 (R)	CAAGAAATTAGTCCTCATCAATC		
RT-PCR	ZAI-NP1	GGACCGCCAAGGTAAAAAATGA	Zaire EBOV NP (268)	48
	ZAI-NP2 (R)	GCATATTGTTGGAGTTGCTTCTCAGC		
Nested RT-PCR[c]	SudZaiNP1 (+) (F)	GAGACAACGGAAGCTAATGC	Sudan and Zaire EBOV NP (150)	50
	SudZaiNP1 (−) (R)	AACGGAAGATCACCATCATG		
	SudZaiNP2 (+) (F)	GGTCAGTTTCTATCCTTTGC		
	SudZaiNP2 (−) (R)	CATGTGTCCAACTGATTGCC		
Real-time qRT-PCR[b]	Unnamed (F)	GAAAGAGCGGCTGGCCAAA	NP ORF region of the Gulu strain of Sudan EBOV (78)	50
	Unnamed (R)	AACGATCTCCAACCTTGATCTTT		
	Unnamed (P)	TGACCGAAGCCATCACGACTGCAT		
Real-time qRT-PCR[b]	EBOGP-1D forward (F)	TGGGCTGAAAAYTGCTACAATC	GP region of Zaire EBOV (112)	18
	EBOGP-1D reverse (R)	CTTTGTGMACATASCGGCAC		
	EBOGP-1DZPrb (P)	CTACCAGCAGCGCCAGACGG		
Real-time qRT-PCR[b]	EBOGP-1D forward (F)	TGGGCTGAAAAYTGCTACAATC	GP region of Sudan EBOV, Boniface strain (112)	18
	EBOGP-1D reverse (R)	CTTTGTGMACATASCGGCAC		
	EBOGP-1DSPrb (P)	TTACCCCCACCGCCGGATG		
Real-time qRT-PCR[b]	ENZ FP (F)	ATGATGGAAGCTACGGCG	NP region of Zaire EBOV (70)	53
	ENZ RP (R)	AGGACCAAGTCATCTGGTGC		
	ENZ P (P)	CCAGAGTTACTCGGAAAACGGCATG		
Real-time qRT-PCR[b]	ENS FP (F)	TTGACCCGTATGATGATGAGAGTA	NP region of Sudan EBOV, Boniface strain (88)	53
	ENS RP (R)	CAAATTGAAGAGATCAAGATCTCCT		
	ENS P (P)	CCTGACTACGAGGATTCGGCTGAAGG		
Real-time qRT-PCR[b]	MN FP (F)	CAATTCCACCTTCAGAAAACTG	NP region of LV MARV,[d] Popp strain (77)	53
	MN RP (R)	GCTAATTTTTCTCGTTTCTGGCT		
	MN P (P)	CACACACAGTCAGACACTAGCCGTCCT		

[a] Primers followed by (F), (R), and (P) indicate forward, reverse, and probe primers, respectively.
[b] qRT-PCR, quantitative RT-PCR.
[c] The primers SudZaiNP1 (+) and SudZaiNP1 (−) are used for the first-round PCR reaction, and the primers SudZaiNP2 (+) and SudZaiNP2 (−) are used for the second-round PCR reaction. The size of the PCR products of 150 bp is expected when the amplicon is generated with the primer set designed for the second-round PCR.
[d] LV MARV, Lake Victoria MARV.

ELISAs using unique monoclonal antibodies to the rNPs of Zaire EBOV, Reston EBOV, and Lake Victoria MARV (20, 39, 44). Although the monoclonal antibodies were produced by immunizing mice with recombinant NPs, the NP-capture ELISA detected not only the rNPs of these viruses but also the authentic EBOV and MARV rNPs. Antigen-capture ELISAs were developed for detecting the NPs of Zaire EBOV, Sudan EBOV, and Reston EBOV (39), that of Reston EBOV alone (20), and that of MARV alone (44). The antigenic regions on the NPs of EBOV and MARV were determined to be located in their carboxy-terminal halves. The carboxy-terminal 110 and 102 amino acids of the NPs of EBOV and MARV, respectively, possess strong antigenicity (45). All the monoclonal antibodies that could be used as capture antibodies in the antigen-capture ELISA format reacted with epitopes within the carboxy-terminal ends of NPs (20, 39, 44). The monoclonal antibodies useful as capture antibodies for rNPs of EBOV and MARV recognized conformational epitopes within the carboxy-terminal ends of NPs, while those for rNP of Reston EBOV recognized linear epitopes (20, 39, 44). Interestingly, the antigen-capture ELISA with capture monoclonal antibodies to the rNP of Reston EBOV (Res2-6C8 and Res2-1D8), which recognize linear epitopes, can detect only rNP of Reston EBOV, but that with capture antibody to rNP of Zaire EBOV (3-3D), which recognizes the conformational epitope, can detect not only the rNP of Zaire EBOV but also rNPs of Sudan, Reston, and Ivory Coast EBOVs (20, 39). Lucht et al. developed an antigen-capture ELISA using a monoclonal antibody to Zaire EBOV GP as the capture antibody and another monoclonal antibody to the same antigen as a detector antibody (35). They also developed an ELISA for the detection of Zaire EBOV VP40, using two monoclonal antibodies to Zaire EBOV VP40 as capture and detector antibodies (34). The EBOV GP-capture ELISA only detected the GP of Zaire EBOV, and the EBOV VP40-capture ELISA detected the VP40s of all four EBOV species. It is considered that EBOV and MARV antigen detection ELISAs are useful for accurate and rapid diagnosis of EHF and MHF. The efficacies of these antigen-capture ELISAs must be evaluated by using patient specimens in a clinical setting and in actual EHF or MHF outbreaks.

Diagnosis by detection of antibodies to EBOV and MARV, including recombinant protein-based antibody detection systems. An indirect immunofluorescence assay developed with authentic virus antigens made from virus-infected cells has been commonly used as a tool for the detection of antibodies to filoviruses (25, 51). It has shown a high sensitivity for antibodies during early convalescence and has been used exten-

TABLE 3. Characteristics of reported antigen-capture ELISAs for filovirus antigens, such as target proteins, capture and detector antibodies, and species of EBOV and MARV that react with each capture antibody

Target protein	Capture antibody	Recognition site of capture antibody	Detector antibody	Reactivity[a]					Reference
				EBOV				LV MARV[b]	
				Zaire	Sudan	Ivory Coast	Reston		
EBOV	Pool of MAbs to Zaire and Sudan EBOVs	Unspecified	Rabbit sera raised to Zaire and Sudan EBOVs	R	R	UK	R	N	28
Zaire EBOV NP	MAb 3-3D to Zaire EBOV	Carboxy-terminal region of Zaire EBOV NP	Rabbit serum raised to Zaire EBOV rNP	R	R	PR	R	N	39
Reston EBOV NP	MAb Res2-6C8 to Reston EBOV NP	Carboxy-terminal region of Reston EBOV NP	Rabbit serum raised to Reston EBOV rNP	N	N	N	R	N	20
	MAb Res2-1D8 to Reston EBOV NP	Carboxy-terminal region of Reston EBOV NP	Rabbit serum raised to Reston EBOV rNP	N	N	N	R	N	20
Zaire EBOV GP	MAb 3B11 to Zaire EBOV GP	Unspecified	POD-labeled MAb 1G12 to Zaire EBOV GP	R	N	N	N	N	35
Zaire EBOV VP40	MAb 2C4 to Zaire EBOV VP40	Unspecified	Biotin-labeled MAb 5F6 to Zaire EBOV VP40	R	R	R	R	N	34
LV MARV[b] NP	MAb 2A7 to MARV NP	Carboxy-terminal region of MARV NP (amino acid residues 632 to 645)	Rabbit serum raised to MARV rNP	N	N	N	N	R	44
	MAb 2H6 to MARV NP	Carboxy-terminal region of MARV NP (amino acid residues 643 to 695)		N	N	N	N	R	

[a] R, UK, N, and PR indicate reactive, unknown, nonreactive, and possibly reactive, respectively.
[b] LV MARV, Lake Victoria MARV.

sively in seroepidemiological surveys of EBOV and MARV antibody prevalence. Recently, an authentic EBOV antigen-based IgG-capture ELISA and IgM-capture ELISA were developed and used as tools for serological diagnosis of EBOV infections (29, 30, 50). The IgM-capture ELISA has great usefulness as a diagnostic tool for EHF. Furthermore, IgG-ELISA is efficacious, not only in diagnosis but also in field investigations of EBOV infections.

It is impossible, however, for institutes without BSL-4 facilities to prepare authentic filovirus antigens from the infectious viruses. Considering the increase in outbreaks of EHF and MHF, the increase in travelers, and the possibility of bioterrorism, diagnostic systems for viral hemorrhagic fevers are necessary even in countries where BSL-4 laboratories are not available. In order to overcome this difficulty, recombinant protein-based diagnostic systems for viral hemorrhagic fevers have been developed by several groups, including ours.

The EBOV and MARV antibody detection systems, in which recombinant proteins of EBOV and MARV are used as antigens, are summarized in Table 4. Prehaud et al. first reported the usefulness of the recombinant nucleoprotein (rNP) and glycoprotein (rGP) of Zaire EBOV strain Gabon 94 for the detection of IgG and IgM antibodies (43), and they studied the efficacy of antibody detection ELISAs developed with EBOV rNP expressed in an *Escherichia coli* system or EBOV rGP expressed in a baculovirus system. All sera collected from seven EHF patients showed positive reactions in both recombinant protein-based antibody-detection ELISAs. Twenty-two control sera showed negative reactions. Thus, the recombinant proteins were confirmed to be useful as antigens for detecting specific antibodies in IgG- and IgM-ELISAs. Saijo et al. further expressed rNPs of Zaire EBOV and MARV (Musoke strain) with a six-His tag at the amino-terminal end, using a baculovirus system (45). The rNP of Zaire EBOV was efficiently expressed. The Zaire EBOV rNP-based IgG-ELISA demonstrated a high sensitivity and specificity, not only for the detection of Zaire EBOV antibodies but also for the detection of Sudan EBOV and Reston EBOV antibodies (45). The NP of MARV, composed of 695 amino acid residues, and the carboxy-terminal half (341 to 695 amino acid residues) possessed strong antigenicity. The carboxy-terminal half of MARV rNP was expressed in an *E. coli* system with a glutathione *S*-transferase tag at the amino-terminal end. IgG-ELISA with the carboxy-terminal half of MARV-rNP was then developed. The IgG-ELISA with the carboxy-terminal half of MARV-rNP also demonstrated a high efficacy and specificity in detecting MARV antibodies (45). The results suggest that recombinant EBOV and MARV rNP-based serological diagnostics are useful for the diagnosis of and seroepidemiological investigations on viral hemorrhagic fevers. Using the same strategy, a Reston EBOV rNP-based IgG-ELISA system was developed (22). EBOV recombinant protein-based IgG-ELISA systems were also developed by other investigators. Groen et al. developed IgG-ELISAs using rNP or recombinant VP35 as antigens. It was demonstrated that a Zaire EBOV rNP-based ELISA detected IgG antibodies, not only to Zaire EBOV but also to other EBOV species, while a Zaire EBOV recombinant VP35 (rVP35)-based ELISA detected IgG antibodies to Zaire EBOV but not to other species. Furthermore, EBOV rNP-based IgG-ELISA was more sensitive in the detection of EBOV antibodies than was EBOV rVP35-based IgG-ELISA. It is noteworthy that the sensitivities and specificities of EBOV recombinant protein-based IgG-ELISAs were evaluated using a large panel of serum samples (26 positive

TABLE 4. Characteristics of recombinant antigen-based antibody detection systems

Method	Origin of antigen	Antigen (amino acid positions)[a]	Expression of recombinant protein	Sensitivity (no. of positive samples/ no. of positive controls)	Specificity (no. of negative samples/ no. of negative controls)	Reference
ELISA	Zaire EBOV (Gabon 94 strain)	rNP	Transformation of *E. coli* with expression vector	9/9	22/22	43
		rGP	Recombinant baculovirus system	9/9	22/22	
	Zaire EBOV	rNP	Recombinant baculovirus system	13/14	50/51	45
		Truncated rNP (361–739)	Transformation of *E. coli* with expression vector	13/14	50/51	
	Zaire EBOV	rNP	Recombinant baculovirus system	24/26	489/500	19
	Zaire EBOV	rVP35	Recombinant baculovirus system	12/26	499/500	19
	Reston EBOV	Truncated rNP (360–739)	Transformation of *E. coli* with expression vector	10/10	72/72	22
	LV MARV[b]	Truncated rNP (341–695)	Transformation of *E. coli* with expression vector	3/3	62/62	45
Indirect immunofluorescence assay	Zaire EBOV	rNP	Infection of HeLa cells with recombinant baculovirus	14/14	47/48	46
	Reston EBOV	rNP	Transfection of HeLa cells with expression vector	16/16	96/96	21

[a] The amino acid positions are counted from the translational initiation codon for each protein.
[b] LV MARV, Lake Victoria MARV.

controls and 500 negative controls) collected from humans and nonhuman primates (19).

The immunofluorescence technique is an alternative method for detecting specific antibodies. Zaire EBOV rNP was expressed in HeLa cells by abortive infection with a recombinant baculovirus that carries the cytomegalovirus immediate-early promoter and the Zaire EBOV NP gene (46). Reston EBOV rNP was also expressed in HeLa cells by transfection with an expression vector, pKS336, carrying the Reston EBOV NP gene (21). The immunofluorescence systems developed with these EBOV rNP-expressing HeLa cells as antigens were confirmed to be highly sensitive and specific (21, 46).

A novel and unique antibody-phage indicator assay for detecting antibodies to the four species of EBOV in any species of animals was also developed (37). A human monoclonal antibody Fab fragment that reacts with an immunodominant epitope on EBOV NP that is conserved in all four EBOV species was established, and a competitive ELISA for detecting antibodies to EBOV was then developed using the Fab fragment. An assay using a phage-antibody spot test, which was able to detect antibodies to EBOV NP in the tested sera by inhibition of the reaction of the Fab antibody to EBOV antigens, was also developed. These assays are considered useful for the diagnosis of EBOV infections and for seroepidemiological studies in the field. Although some species of fruit bats were identified as reservoirs of EBOV (33), the greatest advantage is that this assay can be applied to seroepidemiological research of EBOV infections in a variety of animal species.

CONCLUSIONS

New diagnostic systems for EHF and MHF have been developed recently, using EBOV and MARV recombinant proteins. These include IgG- and IgM-ELISAs, immunofluorescence techniques, and antigen-capture ELISAs with monoclonal antibodies induced by recombinant proteins. These diagnostic systems demonstrate high sensitivities and specificities and therefore are useful for the diagnosis of and epidemiological studies on filovirus infections. Furthermore, preparation of the antigens for these diagnostic systems does not require infectious filoviruses. Thus, the systems are especially advantageous for use in countries where BSL-4 facilities are not available.

REFERENCES

1. **Bausch, D. G., M. Borchert, T. Grein, C. Roth, R. Swanepoel, M. L. Libande, A. Talarmin, E. Bertherat, J. J. Muyembe-Tamfum, B. Tugume, R. Colebunders, K. M. Konde, P. Pirad, L. L. Olinda, G. R. Rodier, P. Campbell, O. Tomori, T. G. Ksiazek, and P. E. Rollin.** 2003. Risk factors for Marburg hemorrhagic fever, Democratic Republic of the Congo. Emerg. Infect. Dis. **9**:1531–1537.
2. **Borio, L., T. Inglesby, C. J. Peters, A. L. Schmaljohn, J. M. Hughes, P. B. Jahrling, T. Ksiazek, K. M. Johnson, A. Meyerhoff, T. O'Toole, M. S. Ascher, J. Bartlett, J. G. Breman, E. M. Eitzen, Jr., M. Hamburg, J. Hauer, D. A. Henderson, R. T. Johnson, G. Kwik, M. Layton, S. Lillibridge, G. J. Nabel, M. T. Osterholm, T. M. Perl, P. Russell, and K. Tonat.** 2002. Hemorrhagic fever viruses as biological weapons: medical and public health management. JAMA **287**:2391–2405.
3. **Bowen, E. T., G. S. Platt, D. I. Simpson, L. B. McArdell, and R. T. Raymond.** 1978. Ebola haemorrhagic fever: experimental infection of monkeys. Trans. R. Soc. Trop. Med. Hyg. **72**:188–191.
4. **Bwaka, M. A., M. J. Bonnet, P. Calain, R. Colebunders, A. De Roo, Y. Guimard, K. R. Katwiki, K. Kibadi, M. A. Kipasa, K. J. Kuvula, B. B. Mapanda, M. Massamba, K. D. Mupapa, J. J. Muyembe-Tamfum, E. Ndaberey, C. J. Peters, P. E. Rollin, E. Van den Enden, and E. Van den Enden.** 1999. Ebola hemorrhagic fever in Kikwit, Democratic Republic of the Congo: clinical observations in 103 patients. J. Infect. Dis. **179**(Suppl. 1):S1–S7.
5. **Centers for Disease Control and Prevention.** 1989. Ebola virus infection in imported primates—Virginia, 1989. Morb. Mortal. Wkly. Rep. **38**:831–832, 837–838.
6. **Centers for Disease Control and Prevention.** 1996. Ebola-Reston virus infection among quarantined nonhuman primates—Texas, 1996. Morb. Mortal. Wkly. Rep. **45**:314–316.

7. **Centers for Disease Control and Prevention.** 2001. Outbreak of Ebola hemorrhagic fever—Uganda, August 2000-January 2001. JAMA **285**:1010–1012.
8. **Conrad, J. L., M. Isaacson, E. B. Smith, H. Wulff, M. Crees, P. Geldenhuys, and J. Johnston.** 1978. Epidemiologic investigation of Marburg virus disease, Southern Africa, 1975. Am. J. Trop. Med. Hyg. **27**:1210–1215.
9. **Drosten, C., S. Gottig, S. Schilling, M. Asper, M. Panning, H. Schmitz, and S. Gunther.** 2002. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. J. Clin. Microbiol. **40**:2323–2330.
10. **Feldmann, H., S. Jones, H. D. Klenk, and H. J. Schnittler.** 2003. Ebola virus: from discovery to vaccine. Nat. Rev. Immunol. **3**:677–685.
11. **Feldmann, H., and M. P. Kiley.** 1999. Classification, structure, and replication of filoviruses. Curr. Top. Microbiol. Immunol. **235**:1–21.
12. **Feldmann, H., W. Slenczka, and H. D. Klenk.** 1996. Emerging and reemerging of filoviruses. Arch. Virol. **11**(Suppl.):77–100.
13. **Feldmann, H., V. E. Volchkov, V. A. Volchkova, U. Stroher, and H. D. Klenk.** 2001. Biosynthesis and role of filoviral glycoproteins. J. Gen. Virol. **82**:2839–2848.
14. **Formenty, P., C. Hatz, B. Le Guenno, A. Stoll, P. Rogenmoser, and A. Widmer.** 1999. Human infection due to Ebola virus, subtype Cote d'Ivoire: clinical and biologic presentation. J. Infect. Dis. **179**(Suppl. 1):S48–S53.
15. **Gear, J. S., G. A. Cassel, A. J. Gear, B. Trappler, L. Clausen, A. M. Meyers, M. C. Kew, T. H. Bothwell, R. Sher, G. B. Miller, J. Schneider, H. J. Koornhof, E. D. Gomperts, M. Isaacson, and J. H. Gear.** 1975. Outbreak of Marburg virus disease in Johannesburg. Br. Med. J. **iv**:489–493.
16. **Geisbert, T. W., and P. B. Jahrling.** 2004. Exotic emerging viral diseases: progress and challenges. Nat. Med. **10**:S110–S121.
17. **Georges-Courbot, M. C., A. Sanchez, C. Y. Lu, S. Baize, E. Leroy, J. Lansout-Soukate, C. Tevi-Benissan, A. J. Georges, S. G. Trappier, S. R. Zaki, R. Swanepoel, P. A. Leman, P. E. Rollin, C. J. Peters, S. T. Nichol, and T. G. Ksiazek.** 1997. Isolation and phylogenetic characterization of Ebola viruses causing different outbreaks in Gabon. Emerg. Infect. Dis. **3**:59–62.
18. **Gibb, T. R., D. A. Norwood, Jr., N. Woollen, and E. A. Henchal.** 2001. Development and evaluation of a fluorogenic 5′ nuclease assay to detect and differentiate between Ebola virus subtypes Zaire and Sudan. J. Clin. Microbiol. **39**:4125–4130.
19. **Groen, J., B. G. van den Hoogen, C. P. Burghoorn-Maas, A. R. Fooks, J. Burton, C. J. Clegg, H. Zeller, and A. D. Osterhaus.** 2003. Serological reactivity of baculovirus-expressed Ebola virus VP35 and nucleoproteins. Microbes Infect. **5**:379–385.
20. **Ikegami, T., M. Niikura, M. Saijo, M. E. Miranda, A. B. Calaor, M. Hernandez, L. P. Acosta, D. L. Manalo, I. Kurane, Y. Yoshikawa, and S. Morikawa.** 2003. Antigen capture enzyme-linked immunosorbent assay for specific detection of Reston Ebola virus nucleoprotein. Clin. Diagn. Lab. Immunol. **10**:552–557.
21. **Ikegami, T., M. Saijo, M. Niikura, M. E. Miranda, A. B. Calaor, M. Hernandez, D. L. Manalo, I. Kurane, Y. Yoshikawa, and S. Morikawa.** 2002. Development of an immunofluorescence method for the detection of antibodies to Ebola virus subtype Reston by the use of recombinant nucleoprotein-expressing HeLa cells. Microbiol. Immunol. **46**:633–638.
22. **Ikegami, T., M. Saijo, M. Niikura, M. E. Miranda, A. B. Calaor, M. Hernandez, D. L. Manalo, I. Kurane, Y. Yoshikawa, and S. Morikawa.** 2003. Immunoglobulin G enzyme-linked immunosorbent assay using truncated nucleoproteins of Reston Ebola virus. Epidemiol. Infect. **130**:533–539.
23. **Jahrling, P. B., T. W. Geisbert, D. W. Dalgard, E. D. Johnson, T. G. Ksiazek, W. C. Hall, and C. J. Peters.** 1990. Preliminary report: isolation of Ebola virus from monkeys imported to USA. Lancet **335**:502–505.
24. **Johnson, E. D., B. K. Johnson, D. Silverstein, P. Tukei, T. W. Geisbert, A. N. Sanchez, and P. B. Jahrling.** 1996. Characterization of a new Marburg virus isolated from a 1987 fatal case in Kenya. Arch. Virol. **11**(Suppl.):101–114.
25. **Johnson, K. M., L. H. Elliott, and D. L. Heymann.** 1981. Preparation of polyvalent viral immunofluorescent intracellular antigens and use in human serosurveys. J. Clin. Microbiol. **14**:527–529.
26. **Johnson, K. M., J. V. Lange, P. A. Webb, and F. A. Murphy.** 1977. Isolation and partial characterisation of a new virus causing acute haemorrhagic fever in Zaire. Lancet **i**:569–571.
27. **Khan, A. S., F. K. Tshioko, D. L. Heymann, B. Le Guenno, P. Nabeth, B. Kerstiens, Y. Fleerackers, P. H. Kilmarx, G. R. Rodier, O. Nkuku, P. E. Rollin, A. Sanchez, S. R. Zaki, R. Swanepoel, O. Tomori, S. T. Nichol, C. J. Peters, J. J. Muyembe-Tamfum, and T. G. Ksiazek.** 1999. The reemergence of Ebola hemorrhagic fever, Democratic Republic of the Congo, 1995. Commission de Lutte contre les Epidemies a Kikwit. J. Infect. Dis. **179**(Suppl. 1): S76–S86.
28. **Ksiazek, T. G., P. E. Rollin, P. B. Jahrling, E. Johnson, D. W. Dalgard, and C. J. Peters.** 1992. Enzyme immunosorbent assay for Ebola virus antigens in tissues of infected primates. J. Clin. Microbiol. **30**:947–950.
29. **Ksiazek, T. G., P. E. Rollin, A. J. Williams, D. S. Bressler, M. L. Martin, R. Swanepoel, F. J. Burt, P. A. Leman, A. S. Khan, A. K. Rowe, R. Mukunu, A. Sanchez, and C. J. Peters.** 1999. Clinical virology of Ebola hemorrhagic fever (EHF): virus, virus antigen, and IgG and IgM antibody findings among EHF patients in Kikwit, Democratic Republic of the Congo, 1995. J. Infect. Dis. **179**(Suppl. 1):S177–S187.
30. **Ksiazek, T. G., C. P. West, P. E. Rollin, P. B. Jahrling, and C. J. Peters.** 1999. ELISA for the detection of antibodies to Ebola viruses. J. Infect. Dis. **179**(Suppl. 1):S192–S198.
31. **Le Guenno, B., P. Formenty, and C. Boesch.** 1999. Ebola virus outbreaks in the Ivory Coast and Liberia, 1994–1995. Curr. Top. Microbiol. Immunol. **235**:77–84.
32. **Leroy, E. M., S. Baize, C. Y. Lu, J. B. McCormick, A. J. Georges, M. C. Georges-Courbot, J. Lansoud-Soukate, and S. P. Fisher-Hoch.** 2000. Diagnosis of Ebola haemorrhagic fever by RT-PCR in an epidemic setting. J. Med. Virol. **60**:463–467.
33. **Leroy, E. M., B. Kumulungui, X. Pourrut, P. Rouquet, A. Hassanin, P. Yaba, A. Delicat, J. T. Paweska, J. P. Gonzalez, and R. Swanepoel.** 2005. Fruit bats as reservoirs of Ebola virus. Nature **438**:575–576.
34. **Lucht, A., R. Grunow, P. Moller, H. Feldmann, and S. Becker.** 2003. Development, characterization and use of monoclonal VP40-antibodies for the detection of Ebola virus. J. Virol. Methods **111**:21–28.
35. **Lucht, A., R. Grunow, C. Otterbein, P. Moller, H. Feldmann, and S. Becker.** 2004. Production of monoclonal antibodies and development of an antigen capture ELISA directed against the envelope glycoprotein GP of Ebola virus. Med. Microbiol. Immunol. (Berlin) **193**:181–187.
36. **Martini, G. A., H. G. Knauff, H. A. Schmidt, G. Mayer, and G. Baltzer.** 1968. On the hitherto unknown, in monkeys originating infectious disease: Marburg virus disease. Dtsch. Med. Wochenschr. **93**:559–571.
37. **Meissner, F., T. Maruyama, M. Frentsch, A. J. Hessell, L. L. Rodriguez, T. W. Geisbert, P. B. Jahrling, D. R. Burton, and P. W. Parren.** 2002. Detection of antibodies against the four subtypes of Ebola virus in sera from any species using a novel antibody-phage indicator assay. Virology **300**:236–243.
38. **Niedrig, M., H. Schmitz, S. Becker, S. Gunther, J. ter Meulen, H. Meyer, H. Ellerbrok, A. Nitsche, H. R. Gelderblom, and C. Drosten.** 2004. First international quality assurance study on the rapid detection of viral agents of bioterrorism. J. Clin. Microbiol. **42**:1753–1755.
39. **Niikura, M., T. Ikegami, M. Saijo, I. Kurane, M. E. Miranda, and S. Morikawa.** 2001. Detection of Ebola viral antigen by enzyme-linked immunosorbent assay using a novel monoclonal antibody to nucleoprotein. J. Clin. Microbiol. **39**:3267–3271.
40. **Niikura, M., T. Ikegami, M. Saijo, T. Kurata, I. Kurane, and S. Morikawa.** 2003. Analysis of linear B-cell epitopes of the nucleoprotein of Ebola virus that distinguish Ebola virus subtypes. Clin. Diagn. Lab. Immunol. **10**:83–87.
41. **Okome-Nkoumou, M., and M. Kombila.** 1999. A case of Ebola virus hemorrhagic fever in Libreville (Gabon), fatal after evacuation to South Africa. Med. Trop. (Mars) **59**:411.
42. **Peters, C. J., and J. W. LeDuc.** 1999. An introduction to Ebola: the virus and the disease. J. Infect. Dis. **179**(Suppl. 1):ix–xvi.
43. **Prehaud, C., E. Hellebrand, D. Coudrier, V. E. Volchkov, V. A. Volchkova, H. Feldmann, B. Le Guenno, and M. Bouloy.** 1998. Recombinant Ebola virus nucleoprotein and glycoprotein (Gabon 94 strain) provide new tools for the detection of human infections. J. Gen. Virol. **79**:2565–2572.
44. **Saijo, M., M. Niikura, A. Maeda, T. Sata, T. Kurata, I. Kurane, and S. Morikawa.** 2005. Characterization of monoclonal antibodies to Marburg virus nucleoprotein (NP) that can be used for NP-capture enzyme-linked immunosorbent assay. J. Med. Virol. **76**:111–118.
45. **Saijo, M., M. Niikura, S. Morikawa, T. G. Ksiazek, R. F. Meyer, C. J. Peters, and I. Kurane.** 2001. Enzyme-linked immunosorbent assays for detection of antibodies to Ebola and Marburg viruses using recombinant nucleoproteins. J. Clin. Microbiol. **39**:1–7.
46. **Saijo, M., M. Niikura, S. Morikawa, and I. Kurane.** 2001. Immunofluorescence method for detection of Ebola virus immunoglobulin G, using HeLa cells which express recombinant nucleoprotein. J. Clin. Microbiol. **39**:776–778.
47. **Sanchez, A., A. S. Khan, S. Zaki, G. J. Nabel, T. G. Ksiazek, and C. J. Peters.** 2001. Filoviridae: Marburg and Ebola viruses, p. 1279–1304. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
48. **Sanchez, A., T. G. Ksiazek, P. E. Rollin, M. E. Miranda, S. G. Trappier, A. S. Khan, C. J. Peters, and S. T. Nichol.** 1999. Detection and molecular characterization of Ebola viruses causing disease in human and nonhuman primates. J. Infect. Dis. **179**(Suppl. 1):S164–S169.
49. **Smith, D. H., B. K. Johnson, M. Isaacson, R. Swanapoel, K. M. Johnson, M. Killey, A. Bagshawe, T. Siongok, and W. K. Keruga.** 1982. Marburg-virus disease in Kenya. Lancet **i**:816–820.
50. **Towner, J. S., P. E. Rollin, D. G. Bausch, A. Sanchez, S. M. Crary, M. Vincent, W. F. Lee, C. F. Spiropoulou, T. G. Ksiazek, M. Lukwiya, F. Kaducu, R. Downing, and S. T. Nichol.** 2004. Rapid diagnosis of Ebola hemorrhagic fever by reverse transcription-PCR in an outbreak setting and assessment of patient viral load as a predictor of outcome. J. Virol. **78**:4330–4341.
51. **van der Groen, G., T. Kurata, and C. Mets.** 1983. Modifications to indirect immunofluorescence tests on Lassa, Marburg, and Ebola material. Lancet **i**:654.

52. **Volchkov, V., V. Volchkova, C. Eckel, H. D. Klenk, M. Bouloy, B. LeGuenno, and H. Feldmann.** 1997. Emergence of subtype Zaire Ebola virus in Gabon. Virology **232**:139–144.
53. **Weidmann, M., E. Muhlberger, and F. T. Hufert.** 2004. Rapid detection protocol for filoviruses. J. Clin. Virol. **30**:94–99.
54. **World Health Organization.** 1978. Ebola haemorrhagic fever in Sudan, 1976. Report of a WHO/International Study Team. Bull. W. H. O. **56**:247–270.
55. **World Health Organization.** 1978. Ebola haemorrhagic fever in Zaire. 1976. Bull. W. H. O. **56**:271–293.
56. **World Health Organization.** 2005. Marburg haemorrhagic fever. Angola. Wkly. Epidemiol. Rec. **80**:158–159.
57. **World Health Organization.** 1996. Outbreak of Ebola haemorrhagic fever in Gabon. Commun. Dis. Rep. CDR Wkly. **6**:75. 78.
58. **World Health Organization.** 2001. Outbreak of Ebola haemorrhagic fever, Uganda, August 2000-January 2001. Wkly. Epidemiol. Rec. **76**:41–46.
59. **World Health Organization.** 1995. Outbreak of Ebola hemorrhagic fever—Zaire, 1995. Epidemiol. Bull. **16**:16.
60. **World Health Organization.** 2005. Outbreak of Marburg virus hemorrhagic fever—Angola, October 1, 2004-March 29, 2005. Morb. Mortal. Wkly. Rep. **54**:308–309.
61. **World Health Organization.** 2003. Outbreak(s) of Ebola haemorrhagic fever in the Republic of the Congo, January-April 2003. Wkly. Epidemiol. Rec. **78**:285–289.
62. **World Health Organization.** 2003. Outbreak(s) of Ebola hemorrhagic fever, Congo and Gabon, October 2001 to July 2002. Can. Commun. Dis. Rep. **29**:129–133.
63. **World Health Organization.** 1992. Viral haemorrhagic fever in imported monkeys. Wkly. Epidemiol. Rec. **67**:142–143.
64. **Zaki, S. R., W. J. Shieh, P. W. Greer, C. S. Goldsmith, T. Ferebee, J. Katshitshi, F. K. Tshioko, M. A. Bwaka, R. Swanepoel, P. Calain, A. S. Khan, E. Lloyd, P. E. Rollin, T. G. Ksiazek, and C. J. Peters.** 1999. A novel immunohistochemical assay for the detection of Ebola virus in skin: implications for diagnosis, spread, and surveillance of Ebola hemorrhagic fever. Commission de Lutte contre les Epidemies a Kikwit. J. Infect. Dis. **179** (Suppl. 1):S36–S47.

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Development of a Real-Time PCR Assay for Detection of *gyrA* Mutations Associated with Reduced Susceptibility to Ciprofloxacin in *Salmonella enterica* Serovar Typhi and Paratyphi A

Masatomo Morita*[,1], Kenitiro Ito[2], Kenji Hirose[1], Hideyuki Takahashi[1], Ken Shimuta[1], Jun Terajima[1], Makoto Ohnishi[1], Makoto Harada[3], Mitsuhiro Matsuzaki[4], Haruo Watanabe[1], and Hidemasa Izumiya[1]

[1]*Department of Bacteriology,* [2]*Infectious Diseases Surveillance Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162–8640, Japan,* [3]*Quarantine and Sanitation Division, Nagoya Quarantine Station, Nagoya, Aichi 455–0045, Japan, and* [4]*Food Hygiene Team, Food Hygiene Analysis Center, Nippon Kaiji Kentei Kyokai, Yokohama, Kanagawa 236–0004, Japan*

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***Abstract*: A real-time PCR assay with the cycling probe method was used to detect mutations at codons 83 and 87 in the DNA gyrase A subunit encoded by *gyrA* in *Salmonella enterica* serovar Typhi and Paratyphi A clinical isolates. The susceptibility estimated from the results of the *gyrA* mutation assay was consistent with that identified by the culture method using an E-test. This assay allows rapid screening of *S. enterica* serovar Typhi and Paratyphi A with reduced susceptibility to ciprofloxacin.**

***Key words*: *Salmonella enterica* serovar Typhi, *Salmonella enterica* serovar Paratyphi A, Real-time PCR, Fluoroquinolone resistance**

Ciprofloxacin (CPFX) is the first-line drug for the treatment of typhoid and paratyphoid fever (13). Recently, resistance to nalidixic acid (NA) has emerged in *S. enterica* serovars Typhi and Paratyphi A, and these NA-resistant strains showed reduced susceptibility to CPFX (9). The spread of strains causing treatment failure with several fluoroquinolones has been reported in developing countries and also in Japan (1, 4, 9, 14, 16). Further, the emergence of fluoroquinolone-resistant *S. enterica* serovars Typhi and Paratyphi A has also been reported (1, 2, 4, 18). CPFX resistance and reduced CPFX susceptibility in typhoid and paratyphoid fevers is a matter of grave concern. The resistance mechanisms against NA and reduced susceptibility to CPFX have been already well studied and reported (3, 6). The acquired resistance was mostly attributed to mutations in the genes encoding DNA gyrase (*gyrA*, *gyrB*). Among them, alterations at codons 83 (Ser) and 87 (Asp) of GyrA are frequently found in clinical isolates with reduced susceptibility to CPFX. Our previous study showed that the CPFX susceptibility of *S. enterica* serovars Typhi and Paratyphi A correlated with the *gyrA* mutations; strains with mutations in both codons 83 and 87 showed resistance to CPFX, a single mutation in either codon 83 or 87 showed reduced susceptibility, and strains with no mutations were susceptible (7).

To assess whether strains show reduced susceptibility, the minimal inhibitory concentration (MIC) should be determined. However, since not all laboratories routinely perform MICs, a disk-diffusion susceptibility test with an NA disk is available for easy screening (5). Currently, it is necessary to develop an alternate DNA-based method which assists in accurate and rapid screening. We previously reported a screening method of *S. enterica* serovars Typhi and Paratyphi A with reduced CPFX susceptibility by PCR-restriction fragment length polymorphism; however, the method involves complicated procedures and requires several hours for analysis (10). In this study, we developed a real-time PCR *gyrA* mutation assay to confirm reduced susceptibility to CPFX using cycling probe technology, which enables highly sensitive detection of the specific

**Address correspondence to* Dr. Masatomo Morita, Department of Bacteriology, National Institute of Infectious Diseases, Toyama 1–23–1, Shinjuku-ku, Tokyo 162–8640, Japan. Fax: +81–3–5285–1163. E-mail: mmorita@nih.go.jp

Abbreviations: Asp, aspartic acid; CPFX, ciprofloxacin; MIC, minimal inhibitory concentration; NA, nalidixic acid; PBS, phosphate buffered saline; PCR, polymerase chain reaction; QRDR, quinolone resistance-determining region; Ser, serine.

sequence in the target gene to identify *gyrA* mutations.

CPFX-resistant and *S. enterica* serovars Typhi and Paratyphi A strains with reduced susceptibility were detected with a CycleavePCR Core Kit (TaKaRa Bio) according to the manufacturer's instructions. The whole genome for template DNA was prepared as previously described (8). Combinations of primers and cycling probes for the detection of mutations in codons 83 and 87 in the quinolone-resistance determining region (QRDR) of *gyrA* are listed in Table 1. The mutation patterns of *gyrA* of *S. enterica* serovars Typhi and Paratyphi A were limited from our previous study (7). All probes were labeled with either FAM or ROX fluorescence at the 5′ end and an eclipse quencher at the 3′ end, which were synthesized by TaKaRa Bio. The PCR reaction and fluorescence detection were performed with a Smart Cycler System (TaKaRa Bio), which detects two kinds of fluorescence at the same time; consequently, FAM- and ROX-labeled probes were used in a single tube during the real-time PCR reactions. The detection of *gyrA* mutations involved two steps. First, DNA from the samples was examined to detect mutations in codons 83 and 87 of GyrA using Wild83 and Wild87 probes, respectively. These two probes were designed to detect TCC in codon 83 and GAC in codon 87 of GyrA, matched with the sequences of ciprofloxacin-susceptible strains. When we observed the fluorescence from both probes, we concluded that the tested strain had no mutation at these sites. Otherwise, we proceeded to the second step using probes for the detection of each mutation (Table 1). The specificities of the primers and probes were examined with strains whose QRDR sequences had been already determined (7). All the fluorescent-labeled cycling probes could detect *gyrA* mutations specifically (Table 2).

Of a total of 42 strains, 24 were *S. enterica* serovar

Table 1. Primers and probes

Primer name	Length	Sequence
gyrA-F	20 mer	GTGACGTAATCGGTAAATAC
gyrA-F2	19 mer	GTAATCGGTAAATACCATC
gyrA-F3	18 mer	GTCGTTGGTGACGTAATC
gyrA-R2	18 mer	CAATAGAACCGAAGTTAC
gyrA-R3	19 mer	CCGTCAATAGAACCGAAG

Probe name	Length	Sequence*	Label	Primer combination
Wild83	11 mer	TGCG (***G***) AATCGC	5′FAM-, 3′Eclipse-label	gyrAF + gyrAR2
Mutant83TTC	11 mer	TGCG (***A***) AATCGC	5′FAM-, 3′Eclipse-label	gyrAF2 + gyrAR3
Mutant83TAC	11 mer	TGCGT (***A***) ATCGC	5′ROX-, 3′Eclipse-label	gyrAF2 + gyrAR3
Wild87	12 mer	TAT (***G***) ACACCATC	5′ROX-, 3′Eclipse-label	gyrAF + gyrAR2
Mutant87TAC	11 mer	TGTATT (***A***) CACC	5′FAM-, 3′Eclipse-label	gyrAF3 + gyrAR2
Mutant87AAC	11 mer	TGTATA (***A***) CACC	5′ROX-, 3′Eclipse-label	gyrAF + gyrAR3
Mutant87GGC	11 mer	TGTATG (***G***) CACC	5′FAM-, 3′Eclipse-label	gyrAF + gyrAR3

PCR conditions were as follows: after holding at 95 C for 10 sec, 40 cycles of denaturation at 95 C for 5 sec, primer annealing at 55 C for 5 sec, and elongation at 72 C for 15 sec. Fluorescence was measured during the 72 C step.

* Bold italic characters in parentheses are RNA.

Table 2. Specificity of primers and probes

Strain name	Codon 83	Codon 87	Screening of mutations in codons 83 and 87		Identification of codon 83 mutations		Identification of codon 87 mutations		
			Wild83 (FAM)	Wild87 (ROX)	83TTC (FAM)	83TAC (ROX)	87GGC (FAM)	87AAC (ROX)	87TAC (FAM)
Ty2 (susceptible)	TCC	GAC	+	+	−	−	−	−	−
990102	TTC	TAC	−	−	+	−	−	−	+
000006	TAC	≡	−	+	−	+	−	−	−
000015	≡	GGC	+	−	−	−	+	−	−
020064	TTC	AAC	−	−	+	−	−	+	−

≡, the same sequences as susceptible strains; +, fluorescence-positive; −, fluorescence-negative.

Typhi, and 18 were *S. enterica* serovar Paratyphi A, and one *Escherichia coli* as a negative control were applied to this system. CPFX susceptibility was estimated using the following criteria: strains with a single mutation were considered to have reduced susceptibility, and strains with double mutations were considered to be resistant, while strains with no mutation were considered susceptible. We compared CPFX susceptibility estimated from a real-time PCR assay with susceptibility determined by a conventional culture method with an

Table 3. The correlation of the results of ciprofloxacin susceptibility by E-test and estimated ciprofloxacin susceptibility by real-time PCR mutation assay

Strain number	Serotype	Results of real-time PCR mutation assay		Estimated ciprofloxacin susceptibility by real-time PCR mutation assay	Results of the sequence determination		Ciprofloxacin susceptibility by E-test
		Codon 83	Codon 87		Codon 83	Codon 87	
050001	TY	TTC	≡	DS	TTC	≡	DS
050003	TY	≡	≡	S	≡	≡	S
050004	TY	≡	≡	S	≡	≡	S
050007	TY	TAC	≡	DS	TAC	≡	DS
050008	TY	≡	≡	S	≡	≡	S
050009	TY	TTC	≡	DS	TTC	≡	DS
050011	TY	TAC	≡	DS	TAC	≡	DS
050012	TY	≡	≡	S	≡	≡	S
050013	TY	≡	≡	S	≡	≡	S
050014	TY	≡	AAC	DS	≡	AAC	DS
050015	TY	TAC	≡	DS	TAC	≡	DS
050018	TY	≡	≡	S	≡	≡	S
050020	TY	≡	≡	S	≡	≡	S
040103	TY	≡	≡	S	≡	≡	S
040104	TY	≡	≡	S	≡	≡	S
040105	TY	≡	≡	S	≡	≡	S
040108	TY	≡	≡	S	≡	≡	S
040109	TY	TTC	≡	DS	TTC	≡	DS
040110	TY	TAC	≡	DS	TAC	≡	DS
040111	TY	TTC	≡	DS	TTC	≡	DS
040119	TY	TTC	≡	DS	TTC	≡	DS
040120	TY	≡	≡	S	≡	≡	S
040121	TY	≡	≡	S	≡	≡	S
040123	TY	≡	≡	S	≡	≡	S
050002	PA	TTC	≡	DS	TTC	≡	DS
050005	PA	TTC	≡	DS	TTC	≡	DS
050006	PA	TTC	≡	DS	TTC	≡	DS
050016	PA	≡	≡	S	≡	≡	S
050017	PA	TTC	≡	DS	TTC	≡	DS
050019	PA	TTC	≡	DS	TTC	≡	DS
040101	PA	TTC	≡	DS	TTC	≡	DS
040102	PA	TTC	≡	DS	TTC	≡	DS
040106	PA	≡	≡	S	≡	≡	S
040107	PA	≡	≡	S	≡	≡	S
040112	PA	TTC	≡	DS	TTC	≡	DS
040113	PA	TTC	≡	DS	TTC	≡	DS
040114	PA	≡	≡	S	≡	≡	S
040115	PA	≡	≡	S	≡	≡	S
040116	PA	TAC	≡	DS	TAC	≡	DS
040117	PA	TTC	≡	DS	TTC	≡	DS
040118	PA	TTC	≡	DS	TTC	≡	DS
040124	PA	≡	≡	S	≡	≡	S
050010	*E.coli*	NA	NA	NA	NA	NA	S

TY, Typhi; PA, Paratyphi A; ≡, the same sequences as susceptible strains; DS, decreased susceptibility; S, susceptible; NA, not applicable.

E-test (AB BIODISK). The MICs of CPFX and NA were determined and strains for quality control, *E. coli* ATCC25922 and *Staphylococcus aureus* ATCC29213, were included in each test (data not shown). The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI [formerly NCCLS]) (12). The breakpoint for the MIC of reduced CPFX susceptibility was defined as ≥0.125 μg/ml and ≤1 μg/ml (1, 15, 17) because reduced susceptibility to CPFX was not detectable using the current CLSI breakpoints. The estimated susceptibilities of all *S. enterica* serovar Typhi and Paratyphi A strains matched the susceptibility determined by the E-test (Table 3). We also determined the sequence of QRDR for confirmation according to our previous study (8). All the sequenced mutations were identical to the mutations predicted from the real-time PCR *gyrA* mutation assay (Table 3).

The emergence of CPFX-resistant or even reduced susceptibility *S. enterica* serovars Typhi and Paratyphi A strains is of great concern because these strains are the cause of treatment failure in typhoid and paratyphoid fever (1, 11). In this context, it is of great importance to know whether the *S. enterica* serovar Typhi and Paratyphi A strains have reduced susceptibility to CPFX (MIC ≥0.125 μg/ml) before medication (1, 15, 17). Hence, a rapid detection system can be useful for screening such strains using novel technologies that have advantages compared to the classical susceptibility tests. Real-time PCR using cycling probes has been evaluated as a rapid detection method for codon 83 and 87 mutations in the QRDR of the *gyrA* gene from CPFX-resistant and reduced susceptibility strains. With this system, *gyrA* mutations can be detected within about 60 min after extracting DNA. The *gyrA* mutations of the *Salmonella* strains tested were correctly identified by this method and the estimated susceptibilities to CPFX from the mutation assay matched the susceptibility from the culture method by E-test. This system might also help to detect reduced fluoroquinolone susceptibility in typhoid fever and paratyphoid fever.

Widespread treatment failure among typhoid and paratyphoid fevers is a grave public health concern and continuous monitoring of antimicrobial resistance is needed. The development and employment of rapid and reproducible molecular methods such as the real-time PCR *gyrA* mutation assay might be helpful to achieve this goal.

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References

1) Aarestrup, F.M., Wiuff, C., Molbak, K., and Threlfall, E.J. 2003. Is it time to change fluoroquinolone breakpoints for *Salmonella* spp.? Antimicrob. Agents Chemother. **47**: 827–829.
2) Adachi, T., Sagara, H., Hirose, K., and Watanabe, H. 2005. Fluoroquinolone-resistant *Salmonella Paratyphi A*. Emerg. Infect. Dis. **11**: 172–174.
3) Brown, J.C., Shanahan, P.M., Jesudason, M.V., Thomson, C.J., and Amyes, S.G. 1996. Mutations responsible for reduced susceptibility to 4-quinolones in clinical isolates of multi-resistant *Salmonella typhi* in India. J. Antimicrob. Chemother. **37**: 891–900.
4) Butt, T., Ahmad, R.N., Mahmood, A., and Zaidi, S. 2003. Ciprofloxacin treatment failure in typhoid fever case, Pakistan. Emerg. Infect. Dis. **9**: 1621–1622.
5) Crump, J.A., Barrett, T.J., Nelson, J.T., and Angulo, F.J. 2003. Reevaluating fluoroquinolone breakpoints for *Salmonella enterica* serotype Typhi and for non-Typhi salmonellae. Clin. Infect. Dis. **37**: 75–81.
6) Griggs, D.J., Gensberg, K., and Piddock, L.J. 1996. Mutations in *gyrA* gene of quinolone-resistant *Salmonella* serotypes isolated from humans and animals. Antimicrob. Agents Chemother. **40**: 1009–1013.
7) Hirose, K., Hashimoto, A., Tamura, K., Kawamura, Y., Ezaki, T., Sagara, H., and Watanabe, H. 2002. DNA sequence analysis of DNA gyrase and DNA topoisomerase IV quinolone resistance-determining regions of *Salmonella enterica* serovar Typhi and serovar Paratyphi A. Antimicrob. Agents Chemother. **46**: 3249–3252.
8) Hirose, K., Itoh, K., Nakajima, H., Kurazono, T., Yamaguchi, M., Moriya, K., Ezaki, T., Kawamura, Y., Tamura, K., and Watanabe, H. 2002. Selective amplification of *tyv* (*rfbE*), *prt* (*rfbS*), *viaB*, and *fliC* genes by multiplex PCR for identification of *Salmonella enterica* serovars Typhi and Paratyphi A. J. Clin. Microbiol. **40**: 633–636.
9) Hirose, K., Tamura, K., Sagara, H., and Watanabe, H. 2001. Antibiotic susceptibilities of *Salmonella enterica* serovar Typhi and *S. enterica* serovar Paratyphi A isolated from patients in Japan. Antimicrob. Agents Chemother. **45**: 956–958.
10) Hirose, K., Tamura, K., and Watanabe, H. 2003. Screening method for *Salmonella enterica* serovar Typhi and serovar Paratyphi A with reduced susceptibility to fluoroquinolones by PCR-restriction fragment length polymorphism. Microbiol. Immunol. **47**: 161–165.
11) Kadhiravan, T., Wig, N., Kapil, A., Kabra, S.K., Renuka, K., and Misra, A. 2005. Clinical outcomes in typhoid fever: adverse impact of infection with nalidixic acid-resistant *Salmonella typhi*. BMC Infect. Dis. **5**: 37.
12) National Committee for Clinical and Laboratory Standards. 2005. Performance standards for antimicrobial susceptibility testing; fifteenth informational supplement M100-S15, National Committee for Clinical and Laboratory Standards, Wayne, Pa.
13) Parry, C.M., Hien, T.T., Dougan, G., White, N.J., and Farrar, J.J. 2002. Typhoid fever. N. Engl. J. Med. **347**: 1770–1782.

14) Rowe, B., Ward, L.R., and Threlfall, E.J. 1995. Ciprofloxacin-resistant *Salmonella typhi* in the UK. Lancet **346**: 1302.
15) Slinger, R., Desjardins, M., McCarthy, A.E., Ramotar, K., Jessamine, P., Guibord, C., and Toye, B. 2004. Suboptimal clinical response to ciprofloxacin in patients with enteric fever due to *Salmonella* spp. with reduced fluoroquinolone susceptibility: a case series. BMC Infect. Dis. **4**: 36.
16) Threlfall, E.J., Skinner, J.A., and Ward, L.R. 2001. Detection of decreased *in vitro* susceptibility to ciprofloxacin in *Salmonella enterica* serotypes Typhi and Paratyphi A. J. Antimicrob. Chemother. **48**: 740–741.
17) Threlfall, E.J., and Ward, L.R. 2001. Decreased susceptibility to ciprofloxacin in *Salmonella enterica* serotype Typhi, United Kingdom. Emerg. Infect. Dis. **7**: 448–450.
18) Threlfall, E.J., Ward, L.R., Skinner, J.A., Smith, H.R., and Lacey, S. 1999. Ciprofloxacin-resistant *Salmonella typhi* and treatment failure. Lancet **353**: 1590–1591.

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Significant passive protective effect against anthrax by antibody to *Bacillus anthracis* inactivated spores that lack two virulence plasmids

Jargalsaikhan Enkhtuya,[1] Keiko Kawamoto,[1] Yoshiyasu Kobayashi,[2] Ikuo Uchida,[3] Neeraj Rana[1] and Sou-ichi Makino[1]

Correspondence
Keiko Kawamoto
kkeiko@obihiro.ac.jp

[1,2]Laboratory of Food Microbiology and Immunology, Research Center for Animal Hygiene and Food Safety[1] and Department of Pathobiological Science[2], Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080-8555, Japan

[3]Hokkaido Research Station, National Institute of Animal Health, Hitsujigaoka 4, Toyohira-Ku, Sapporo, Hokkaido 062-0045, Japan

The protective-antigen (PA)-based cell-free vaccine is the only vaccine licensed for use against *Bacillus anthracis* infection in humans. Although the PA shows strong immunogenicity, the capsule or spore-associated somatic antigens may be important as additional vaccine targets for full protection against anthrax. In this study, the protective effect of spore-associated antigens against *B. anthracis* infection was determined. Rabbits were immunized with formalin-fixed spores of a non-toxigenic unencapsulated *B. anthracis* strain that lacked the two virulence plasmids pXO1 and pXO2, and the protective effects of the immune antibody were evaluated. Immunostaining and Western blot analysis revealed that the anti-*B. anthracis* (anti-BA)-spore IgG specifically bound to the surface of spores or endospores of *B. anthracis*, but not to vegetative cells, or closely related *Bacillus* species, such as *Bacillus cereus*, *Bacillus subtilis* and *Bacillus thuringiensis*. Passively transferred anti-BA-spore IgG protected mice from intraperitoneal challenge with a lethal dose of fully virulent *B. anthracis* spores, and increased the survival rate in a dose-dependent manner. Pre-incubation of spores with antibody also reduced their infectivity in a dose-dependent manner. The number of bacteria (c.f.u.) in spleens and livers of infected mice was significantly lower in antibody-treated mice than in untreated mice. Treatment with anti-BA-spore IgG also inhibited the germination of spores in J774.1 macrophages, suggesting that opsonization of spores promotes phagocytosis and subsequent killing by macrophages. These results indicate the usefulness of spore surface antigens as vaccine targets. In combination with major virulence factors such as the PA, spore-associated antigens may offer a safer and more effective multicomponent vaccine for *B. anthracis* infection.

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INTRODUCTION

Bacillus anthracis, a Gram-positive spore-forming bacterium, is the causative agent of anthrax. Dormant spores are highly resistant to stressful conditions, and they are able to survive for decades in the environment. Pathogenesis of inhalational and cutaneous anthrax includes the entry of *B. anthracis* spores into the host, followed by germination, bacterial multiplication, dissemination and toxin production. Macrophages play an important role in the early stages of anthrax, and constitute a primary site for spore germination (Guidi-Rontani *et al.*, 1999). However, the spore germination mechanism in the presence of host immunity has not yet been fully elucidated. The virulence of *B. anthracis* infection is mainly mediated by two virulence plasmids, termed pXO1 and pXO2 (Okinaka *et al.*, 1999a, b). pXO1 encodes three toxin components: lethal factor (LF), (o)edema factor (EF) and protective antigen (PA). pXO2 encodes an anti-phagocytic capsule that consists of poly-γ-D-glutamic acid polymer (Makino *et al.*, 1988; Mikesell *et al.*, 1983; Uchida *et al.*, 1986). The PA binds to the surface of receptors expressed on target cells, and serves as a carrier for LF and EF to deliver toxins into the host cell cytosol (Nourez *et al.*, 2002).

The natural course of anthrax infection commonly occurs in wild and domestic animals through the uptake of spores that remain viable in contaminated soil for many years (Turnbull, 2002). Humans are an occasional host, but may also become infected upon exposure to spores from

Abbreviations: anti-BA, anti-*Bacillus anthracis*; AVA, anthrax vaccine adsorbed; EF, (o)edema factor; i.p., intraperitoneal(ly); LF, lethal factor; PA, protective antigen; p.i., post-infection.

infected animals or their tissues (Mock & Fouet, 2001). However, after the anthrax attack via the US postal system in 2001, the potential use of spores as bioweapons has been a public concern, which has resulted in a heightened interest in the pathogenesis of anthrax, and development of a vaccine against it (Inglesby *et al.*, 2002; Jernigan *et al.*, 2001, 2002).

The anthrax vaccines currently available are: anthrax vaccine adsorbed (AVA) for humans; live attenuated spore vaccines derived from encapsulated strains, for veterinary use only (Brey, 2005); and the UK anthrax vaccine, the anthrax vaccine precipitated, which has been in use for over 40 years (Williamson *et al.*, 2005). The live spore STI vaccine has been used in Russia for many years in humans (Romanov, 1980). Many researchers have demonstrated that high titres of anti-PA antibody also give passive protection in rabbits, guinea pigs and mice (Beedham *et al.*, 2001; Kobiler *et al.*, 2002; Pitt *et al.*, 2001). Antibodies to the PA may neutralize toxin activity by blocking the binding of the PA to its receptor, and/or by the formation of a toxin complex. Due to its function and immunogenicity, the PA has been an attractive target for vaccine development. Protection can also be induced by immunization with purified PA, the whole organism, or DNA plasmids carrying recombinant PA (Gu *et al.*, 1999; Iacono-Connors *et al.*, 1991; Ramirez *et al.*, 2002; Rhie *et al.*, 2005; Vodka & Leppla, 1983; Watson *et al.*, 2005). However, more effective vaccines giving full protection against anthrax may require additional bacterial components, including the capsule or somatic antigens.

In this study, we elucidate the immunogenicity of spore-associated antigens. To eliminate the possible involvement of the major virulence factors, such as the PA, LF, EF and capsule, we immunized the rabbits with formalin-fixed spores of the *B. anthracis* Pasteur II strain, which lacks the pXO1 and pXO2 virulence plasmids. Purified antibodies from the rabbit serum recognized the surface molecules expressed on the spores of both the fully virulent and the plasmidless *B. anthracis* strains, but not on those of other closely related *Bacillus* species. In immunoblot analysis, several spore-specific proteins were detected using anti-BA-spore IgG. The passive transfer of purified anti-BA-spore IgG into naive mice conferred protection against lethal doses of *B. anthracis* challenge, and the effect was dose dependent. Treatment with anti-BA-spore IgG also promoted the killing of the bacteria by macrophages, and caused a reduction in the number of germinated spores. These results suggest that somatic antigens expressed on the spore surface may be useful for the development of a new and more effective anthrax vaccine.

METHODS

Bacterial strains and spore preparation. *B. anthracis* Pasteur II (Uchida *et al.*, 1993), a fully virulent ($pXO1^+$, $pXO2^+$) strain, its derivative plasmidless ($pXO1^-$, $pXO2^-$) strain, and the Sterne ($pXO1^+$, $pXO2^-$) strain, were used in this study. Other *Bacillus* species, including *B. cereus*, *B. subtilis* and *B. thuringiensis* strains maintained in our laboratory, were also used. For spore preparation, fresh cultures grown in Luria–Bertani broth (LB broth) were spread on a low-nutrient agar [0·8 % nutrient broth and 1 % yeast extract (Difco), 0·05 % $MgSO_4.7H_2O$, 0·2 % KCl, 1·6 % agar, 0·1 % 0·1 M $MnSO_4$, 0·1 % glucose, 0·1 % 1 M $Ca(NO_3)_2$, 0·1 % 1 mM $FeSO_4$], and incubated at 32 °C for 7 days. After 7 days, the spores were collected, and washed 10 times with chilled sterile distilled water. To kill the vegetative cells, the suspension was incubated at 85 °C for 20 min. The suspension was then washed several times with distilled water, and the spores were examined by Gram staining. Spore numbers were enumerated using the plate counting method, and the spores were preserved at 4 °C in sterile distilled water until use.

Antibody production and purification. *B. anthracis* ($pXO1^-$, $pXO2^-$) spores were inactivated overnight with 4 % paraformaldehyde (Sigma), and washed twice with PBS. After ensuring that spores had been inactivated, by testing for growth on LB agar at 37 °C for 24 h, they were used as an immunogen. Two Japanese white rabbits (Charles River), weighing about 2 kg each, were subcutaneously immunized four times, at 2 week intervals, with a mixture containing 10^7 spores and Freund's complete or incomplete adjuvant (Sigma) to induce sufficient antibody production. The antibody titre was monitored by ELISA. Rabbit blood was collected 2 days after the last immunization, and IgG was purified from immune serum by using a protein G-conjugated column (Mab Trap kit; Amersham Biosciences).

Specificity of anti-BA-spore IgG. The spores prepared from *Bacillus* species were fixed overnight with 1 % paraformaldehyde on microscopic glass slides until completely dry. The inactivated and immobilized spores were treated with PBS containing 0·5 % Tween 20 (PBS-T) and 3 % skim milk to block non-specific binding sites, and washed three times with PBS-T. The slides were incubated with 100 µl anti-BA-spore IgG (10 µg ml^{-1}) for 1 h at room temperature in a humid chamber. This was followed by washing, and 100 µl Alexa Fluor 488-conjugated goat anti-rabbit IgG at a dilution of 1 : 1000 (Molecular Probes) was added to each slide, which was then incubated for 30 min in a dark place. After washing, the slides were mounted in aqueous mounting medium containing an anti-fading agent (Biomedia), and sealed with nail polish. The samples were observed under a fluorescence microscope (Olympus BX51; Opelco), and the images were visualized using DP70-BSW software.

Spore protein preparation, and Western blot assay. Spore protein samples were prepared as described by Kim *et al.* (2004). Briefly, the spores were suspended in UDS buffer [6 M urea (Sigma), 50 mM DTT and 1 % SDS (Wako)], and incubated at 37 °C for 20 min. The suspensions were centrifuged at 10 000 ***g*** for 6 min, and the supernatants were collected. The incubation was repeated, and the supernatants were pooled. The protein concentrations of samples were measured by spectrophotometry at A_{280}. The same amounts of samples (< 10 µg) were mixed with sample buffer, and, after boiling, the proteins were separated in an electrophoretic cell (Mini Protean 3; Bio-Rad) at a constant current (20 mA per gel) for 1 h. The separated proteins were transferred to a PVDF membrane (Millipore) in an electrophoretic semi-dry transfer cell (Trans-Blot SD; Bio-Rad) at 15 V for 1 h. The PVDF membrane was blocked by overnight incubation in PBS-T containing 3 % skim milk. After washing three times with PBS-T, the membrane was incubated at room temperature for 1 h with anti-BA-spore IgG diluted 1 : 40 000 in PBS-T containing 0·3 % skim milk, and washed as described above. The membrane was then incubated at room temperature for 1 h with peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences) diluted 1 : 10 000 in PBS-T containing 0·3 % skim milk. The blots were washed in PBS-T, and bound peroxidase enzyme was detected with the ECL-Plus Western blotting detection reagent (Amersham Biosciences).

Mouse infection. Four-week-old female ICR mice (Clea) were separated into groups containing equal numbers, and were injected intraperitoneally (i.p.) with 200 µl PBS containing 5×10^3 fully virulent *B. anthracis* spores, in the presence or absence of anti-BA-spore IgG. Before administration, the spores were incubated with a single dose of 0·01, 0·1 or 0·5 mg anti-BA-spore IgG per mouse, or PBS as a control, on ice for 30 min, and washed twice with sterile PBS to remove excess antibodies. The infection was performed in two separate experiments, and survival of the mice was monitored twice a day for up to 8 days post-infection (p.i.). In order to detect bacteria in the spleens and livers of mice, at 44, 68, 92, 116 and 140 h p.i. (five mice per group), the spleens and livers were isolated, and homogenized in sterile distilled water. Appropriately diluted homogenates were plated on tryptic soy agar (Difco) for enumeration of bacteria.

Passive immunization. Six-week-old female ICR mice (Clea) were injected with anti-BA-spore IgG or PBS at different times pre- and post-challenge (i.p.) of 5×10^3 fully virulent *B. anthracis* spores. For the pre-exposure prophylaxis, the mice (seven per group) were administered i.p. with a single dose of 0·01, 0·1 or 0·5 mg anti-BA-spore IgG at 30–40 min prior to spore challenge. PBS was given to the control groups. To evaluate the post-exposure efficacy, mice were challenged with the spores, and then injected i.p. with a single dose 0·01, 0·1 or 0·5 mg anti-BA-spore IgG, or PBS as a control, at 24, 36 and 48 h post challenge. All mice were observed twice daily for 8 days post-challenge.

Histopathology. The organs of mice that had been infected with pre-incubated anthrax spores, in the presence or absence of IgG (a single dose of 0·01, 0·1 or 0·5 mg per mouse), as described above, were used for histopathological examination. The mice were sacrificed at 44 h p.i., and the spleen and kidneys were removed and then fixed in 4% paraformaldehyde for 7 days. The sectioned tissues (5 µm) were stained with haematoxylin and eosin, by using conventional protocols, and observed under the microscope (Olympus BX51; Opelco). The images were visualized using DP70-BSW software.

Effect of anti-BA-spore IgG on macrophage and spore interaction. The J774.1 cell line (ATCC TIB-106) was cultured for 3–4 days at 37 °C in 5% CO_2 in RPMI medium containing 10% fetal bovine serum (Sigma), 200 mM L-glutamine, 50 U penicillin ml^{-1} and 50 µg streptomycin ml^{-1}. The cells were then washed, suspended in sterile PBS, and 2×10^5 cells dispensed into a siliconized microcentrifuge tube (Fisherbrand). The spores were incubated on ice for 30 min with 0·01, 0·1 or 0·5 mg anti-BA-spore IgG or PBS, and washed twice with sterile PBS to remove excess antibodies. Then they were allowed to interact with macrophages (m.o.i. 1:10) for 30 min at 37 °C in 5% CO_2. The cells were then washed three or four times with PBS, and suspended in RPMI medium containing 10 µg gentamicin ml^{-1}, and incubated for 30 min at 37 °C in 5% CO_2. After each washing step, the supernatants were collected for counting of non-interacting spores. After completion of the incubation, the samples were heat inactivated at 75 °C for 30 min to kill any vegetative bacilli. Intracellular ungerminated spores were then enumerated using the plate-counting method and tryptic soy agar plates. Enumeration of spores that had interacted with macrophages was also performed.

RESULTS

Specificity of anti-BA-spore IgG

We evaluated the specificity of rabbit anti-BA-spore IgG by fluorescence immunostaining. As shown in Fig. 1, distinct fluorescence, indicating the specific binding of antibody, was observed in the spores of the pXO1$^-$, pXO2$^-$ strain and the pXO1$^+$, pXO2$^+$ strain. Fluorescence was not detected in spores of *B. cereus*, *B. subtilis* and *B. thuringiensis* (data not shown). Immunofluorescence of anti-BA-spore IgG was localized on the surface of *B. anthracis* spores and endospores, but not in the vegetative cells. These results suggest that anti-BA-spore IgG reacts specifically with *B. anthracis* spores.

To identify the molecules recognized by the anti-BA-spore IgG, spore protein samples were prepared from three strains (pXO1$^-$, pXO2$^-$ strain, pXO1$^+$, pXO2$^+$ Pasteur II strain and pXO1$^+$, pXO2$^-$ Sterne strain), and subjected to immunoblotting. As shown in Fig. 2, eight protein bands were found to be common to all the spores; however, the intensity of each band was different among the strains. In spores of the pXO1$^-$, pXO2 strain, two bands with

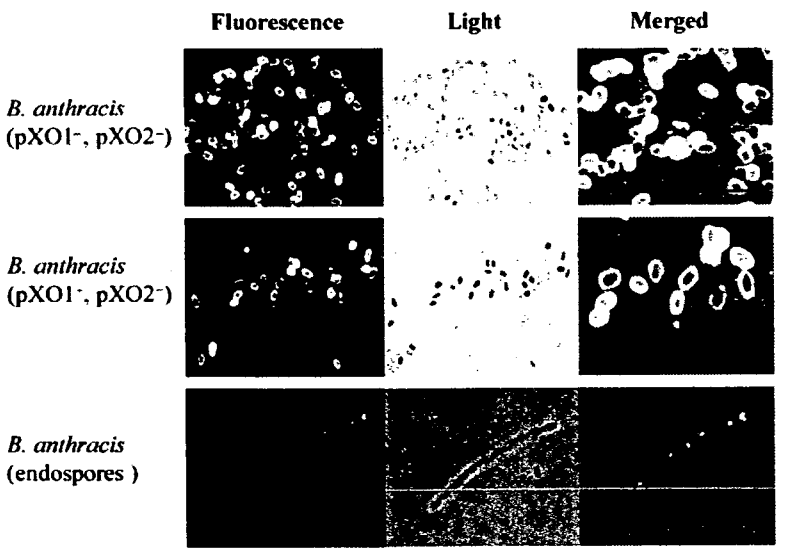

Fig. 1. Immunostaining of *B. anthracis* spores and endospores in a vegetative cell. Spores of two strains of *B. anthracis* (pXO1$^-$, pXO2$^-$ strain, and pXO1$^+$, pXO2$^+$ strain) were inactivated and immobilized by overnight fixation with 1% paraformaldehyde, and then immunostained with affinity-purified rabbit anti-BA-spore IgG. Fluorescence and light images were taken at ×100 magnification, while merged images were enlarged. The images were visualized using DP70-BSW software. Bars, 50 µm.

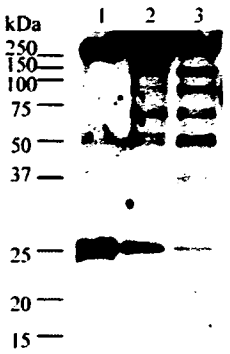

Fig. 2. Western blot analysis of the *B. anthracis* spore proteins recognized by anti-BA-spore IgG. Whole spore proteins were prepared in UDS lysis buffer, and separated on a 12% SDS gel. Lanes: 1, pXO1$^-$, pXO2$^-$; 2, pXO1$^+$, pXO2$^+$ Pasteur II; 3, pXO1$^+$, pXO2$^-$ Sterne.

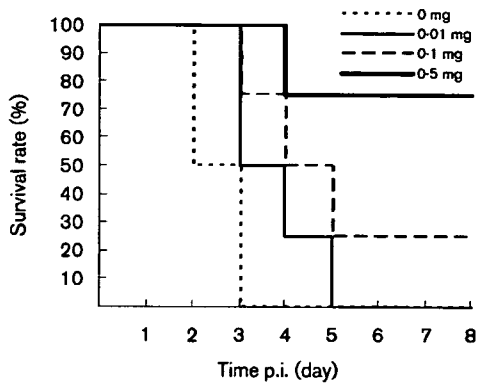

Fig. 3. Survival rates of mice with *B. anthracis* infection. The data show the combined results of three independent experiments. Mice (12 per group) were infected i.p. with a lethal dose of virulent *B. anthracis* spores (5×10^3 spores per mouse) that that had been pre-incubated with 0, 0·01, 0·1 or 0·5 mg anti-BA-spore IgG. The survival of mice was monitored twice daily for up to 8 days p.i.

molecular masses of >250 and 25 kDa were prominent, while the other six bands were faint (lane 1). In the pXO1$^+$, pXO2$^+$ Pasteur II strain, and the pXO1$^+$, pXO2 Sterne strain, the anti-BA-spore IgG identified essentially the same proteins as in the pXO1$^-$, pXO2$^-$ strain. However, the bands that showed up weakly for the pXO1$^-$, pXO2$^-$ strain were stronger in the Pasteur II and Sterne strains, despite the protein samples being the same concentration for all the strains. In the Sterne strain, the band corresponding to 25 kDa was weaker than in the other two strains, and additional protein bands of about 37 and 15 kDa were detected.

Protective effect of anti-BA-spore IgG against anthrax infection in mice

To examine the protective effect of anti-BA-spore IgG in mice, 12 mice in each group were injected i.p. with a lethal dose of *B. anthracis* spores (5×10^3), and various concentrations of the anti-BA-spore IgG (a single dose of 0·01, 0·1 and 0·5 mg per mouse). All the mice in the control group died within 3 days of the anthrax spore challenge (Fig. 3). In contrast, the antibody-treated mice survived longer, and treatment with the anti-BA-spore IgG resulted in a significant decrease in mortality. The experiment was performed in triplicate, and the results were combined.

Doses of 0·5 and 0·1 mg IgG produced survival rates of 75 and 25 %, respectively, at 8 days p.i. The results suggest that antibody specific to *B. anthracis* spores has a protective effect against anthrax in mice. The surviving mice were sacrificed, and bacteria were not detected in the liver and spleen.

We examined the numbers of bacteria in the livers and spleens of the sacrificed mice during infection (Fig. 4). When the mice were inoculated with 5×10^3 spores i.p., the number of bacilli in the livers and spleens reached levels of approximately 10^9 or 10^8 c.f.u. g^{-1} at 44 h p.i. in the group infected with spores, and none of the mice had survived on day 3 p.i. In contrast, the numbers of c.f.u. in the organs of the antibody-treated mice were reduced in a dose-dependent manner. The difference in mortality between the control and antibody-treated mice paralleled the change in the bacterial count in the organs. In the spleens and livers of surviving mice treated with 0·1 or 0·5 mg antibody, it was found that all bacteria had been eliminated by day 6 p.i.

Histopathological examination of paraformaldehyde-fixed paraffin-embedded spleens and kidneys of infected mice was also conducted. Infected mice that had not had antibody

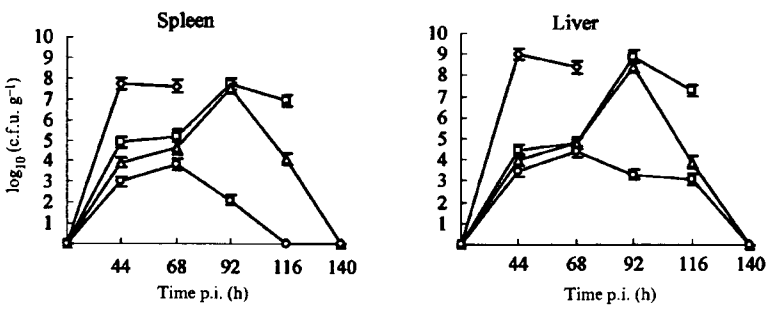

Fig. 4. Inhibitory effect of anti-BA-spore IgG on bacterial proliferation. Mice (five per group) were infected i.p. with a lethal dose of virulent *B. anthracis* spores (5×10^3 spores per mouse) that had been pre-incubated with 0 (◇), 0·01 (□), 0·1 (△) or 0·5 mg (○) anti-BA-spore IgG. From 44 h p.i., the spleens and livers were removed (see Methods) for bacterial counts. The data are shown as means (±SD).

treatment showed infectious splenitis to varying degrees (Fig. 5a). In the spleen, at 44 h p.i., numerous karyorrhectic lymphocytes were diffusely observed in the splenic follicules and red pulp. Abundant starry sky figures by tingible macrophages were also detected in the splenic follicules. Furthermore, there was considerable lymphoid depletion with congestion, dilation of splenic sinuses, and neutrophil infiltration in the splenic red pulp, especially at the perifollicular areas. Numerous rod-shaped bacilli were also identified by haematoxylin and eosin stain, most often abundant in the perifollicular areas. In the kidneys, numerous bacilli were noted in congested blood capillaries at the glomerular tufts and interstitium (Fig. 5c). However, these pathological findings were not as marked as those in the organs of the antibody-treated mice, including those in the low-dose antibody-treated group (0·01 mg). Furthermore, clusters of bacilli were not observed in any of the tissues from the antibody-treated mice (Fig. 5b, d).

Passive protective effect of anti-BA-spore IgG against *B. anthracis* spore challenge in mice

The ability of the anti-BA-spore IgG to protect against anthrax was tested in mice pre- and post-challenge with *B. anthracis* spores. Pre-challenge administration of anti-BA-spore IgG was performed 30–40 min prior to anthrax spore challenge: mice ($n=7$) were given a single i.p. injection of 0·01, 0·1 or 0·5 mg IgG, and the control group ($n=7$) received 100 µl PBS. In the control group, all mice had died by day 3 post-challenge (Fig. 6a). In contrast, the mice that had received anti-BA spore IgG, including those that received a single dose of 0·01 mg only, survived longer than the control mice. Anti-BA-spore IgG at 0·1 or 0·5 mg prolonged the lives of the mice, and produced survival rates of up to 14·2 and 42·8 %, respectively. The post-challenge administration of anti-BA-spore IgG was performed 24 ($n=5$), 36 ($n=6$) and 48 ($n=8$) h after anthrax spore challenge: mice were given a single i.p. injection of 0·01, 0·1 or 0·5 mg IgG, and the control group received 100 µl PBS. When anti-BA-spore IgG administration was performed 24 h post-anthrax-spore challenge, the mice in the control group had died within 3 days, while the mice in the IgG-

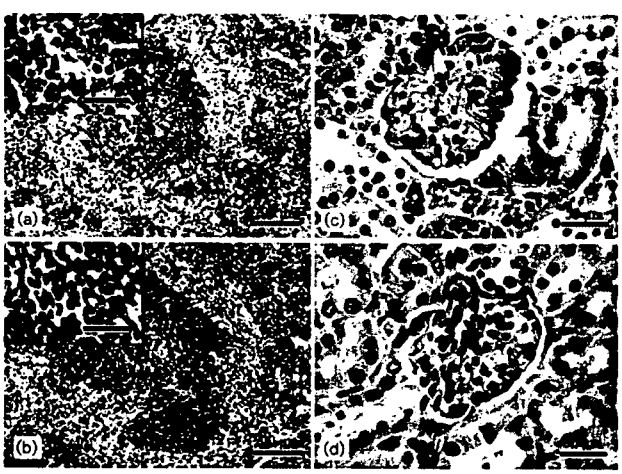

Fig. 5. Photomicrographs of spleens (a, b) and kidneys (c, d) from mice infected with *B. anthracis* spores (5×10^3 spores per mouse). (a, c) Tissues from a control mouse infected with spores; (b, d) tissues from a mouse infected with spores, and treated with 0·5 mg anti-BA-spore IgG. In sections from the control mouse, necrotizing splenitis (a) and dilation of glomerular capillaries (c) were evident, with clusters of bacilli [a (inset), c]. In sections from the antibody-treated mouse, there were no pathological findings (b, d), and clusters of bacilli were not observed [b (inset), d]. The sections were stained with haematoxylin and eosin, and images were visualized using DP70-BSW software.

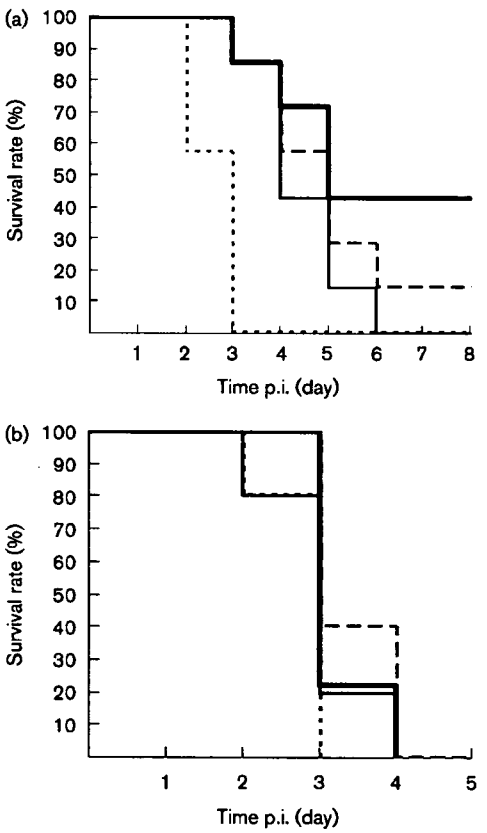

Fig. 6. (a) Survival rates of mice injected with anti-BA-spore IgG prior to *B. anthracis* spore challenge. Mice (seven per group) were injected with a single dose (i.p.) of 0·01 (continuous line), 0·1 (dashed line) or 0·5 mg (bold continuous line) anti-BA-spore IgG, or PBS as a control (dotted line), 30–40 min prior to spore challenge (5×10^3 spores per mouse). Experiments were performed twice independently. (b) Survival rates of mice treated with anti-BA-spore IgG 24 h after exposure to *B. anthracis* spores. Mice (five per group) were challenged i.p. with a lethal dose of virulent *B. anthracis* spores (5×10^3 spores per mouse), before receiving 0·01 (continuous line), 0·1 (dashed line) or 0·5 mg (bold continuous line) anti-BA-spore IgG, or PBS as a control (dotted line).

treated groups survived a day longer (Fig. 6b). When antibody (0·01, 0·1 or 0·5 mg) administration was performed at 36 and 48 h post-anthrax-spore challenge, all mice treated with the anti-BA-spore IgG had died by day 3, regardless of which group they were in, and this was the same for the control mice (data not shown).

Inhibitory effect of anti-BA-spore IgG on germination

Macrophages may play a dual role in *B. anthracis* infection: as an intracellular niche permissive for spore germination, and as effector cells for clearing spores. Therefore, we studied the effect of anti-BA-spore IgG on the germination of spores in macrophages. J774.1 macrophage cells ($2·5 \times 10^5$) were cultured with *B. anthracis* spores (m.o.i. 1 : 10) in the presence of various amounts of anti-BA-spore IgG (0·01, 0·1 and 0·5 mg). In the untreated cells, intracellular ungerminated spores were approximately 10 % of the total interacting spores. However, in the cells treated with various amounts of anti-BA-spore IgG, numbers of ungerminated spores were significantly higher than for the untreated group. In the 0·01 mg IgG-treated group, ungerminated spores were approximately 15 % of the total interacting spores, while 27 and 50 % of the total interacting spores were not able to germinate in groups treated with 0·1 and 0·5 mg IgG, respectively. The results indicate that the anti-BA-spore IgG has an inhibitory effect on spore germination in a dose-dependent manner.

DISCUSSION

Studies on *B. anthracis* by other authors have demonstrated that the efficacy of PA-based vaccines is not satisfactory when compared with the live attenuated spore vaccine (Little & Knudson, 1986; Welkos & Friedlander, 1988). Vaccination studies with live or attenuated pXO1$^+$, pXO2$^-$ spores have shown that immune responses against the *B. anthracis* spore and PA lead to greatly improved protection in comparison with responses to PA alone (Brossier *et al.*, 2002; Cohen *et al.*, 2000; Welkos & Friedlander 1988). This could be because PA-based vaccines target toxaemia rather than bacteraemia. The capsular material itself has been shown to be poorly immunogenic, and thus not suitable as a vaccine component; however, conjugation of poly-γ-D-glutamic acid as protein carrier makes it more effective as a vaccine (Joyce *et al.*, 2006). These findings indicate the complexity of anthrax pathogenesis, and also suggest that PA, capsule and somatic antigens are essential for full protection against the disease. Chromosomally encoded molecules expressed on the spore surface have been considered as possible candidates for vaccine development; however, there have been no reports evaluating the immunogenicity of spores derived from virulence-plasmid-negative *B. anthracis* strains. Since the efficacy of a spore vaccine has been demonstrated for strains harbouring pXO1$^+$ (Brossier *et al.*, 2002), it is difficult to elucidate the possible contribution of spore antigens in protective immunity. Thus, to assess the immunogenicity of spore surface proteins, we used pXO1$^-$, pXO2$^-$ *B. anthracis* spores, and conducted experiments to evaluate the antibodies obtained from the animals that had been immunized with formalin-fixed, plasmidless spores. The immune antibody specifically reacted with *B. anthracis* spores or endospores, but not with vegetative cells, and it protected mice from lethal challenge with fully virulent *B. anthracis* in the passive immunization test (Fig. 6a). We also examined whether antibodies in the anti-BA-spore polyclonal IgG could bind to PA, but no reactivity was observed in an ELISA (data not shown).

The anti-BA-spore IgG specifically reacted with the surface of *B. anthracis* spores, but not with spores of related bacilli, such as *B. cereus*, *B subtilis* and *B. thuringiensis*, as shown by immunostaining (Fig. 1). As shown in Fig. 2, the polyclonal antibody recognized some spore proteins of pXO1$^-$, pXO2$^-$ *B. anthracis* spores, as well as those of the fully virulent pXO1$^+$, pXO2$^+$ Pasteur II strain, and the pXO1$^+$, pXO$^-$ Sterne strain. These results indicate that these spore-associated immunogenic antigens may be encoded by genes on the *B. anthracis* chromosome, and that they may be present on the spore surface. The two spore proteins that appeared as the strongest bands in immunoblots for the strains in this study were of a similar molecular size to those identified in other reports. For example, the band a with molecular mass of 250 kDa is likely to be the collagen-like spore surface glycoprotein (BclA), which is a structural component of the filaments of the hairy nap, and is highly immunogenic (Sylvestre *et al.*, 2002). We also detected a band in the vicinity of 25 kDa that may correspond to a 24·6 kDa spore surface protein predicted to be iron/manganese superoxide dismutase, based on its amino-terminal sequence (Read *et al.*, 2002; Steichen *et al.*, 2003).

Some hypothetical proteins have been detected in dormant and germinating spores of the Sterne 34F2 strain, and these include one with a molecular mass of 25·5 kDa (Huang *et al.*, 2004). Also, in a recent investigation, *B. anthracis* spore-associated proteins have been identified in reactions with the sera of humans immunized with AVA vaccine, and the molecular masses of some of the proteins are close to 25 kDa, for example, transdolase, thiazole biosynthesis protein and rRNA adenine dimethylase (Kudva *et al.*, 2005). The other protein bands detected in the immunoblot analysis (Fig. 2) were of weak intensity for the pXO1$^-$, pXO2$^-$ strain, and corresponded to 50, 70, 90 and 110 kDa. However, the bands were stronger for the pXO1$^+$, pXO2$^+$ and pXO1$^+$, pXO2$^-$ virulent *B. anthracis* strains, and this result might suggest that the two virulence plasmids (pXO1 and pXO2) contain genes that control the production of these proteins. In other studies of spore-associated proteins, several identified proteins have been found to have similar sizes to those found in this study, including spoOB-associated GTP-binding protein, sensory box histidine kinase, immuno-inhibitor A, and S-layer homology domain, with molecular masses of 50, 70, 90 and