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An Ocular Infection Model Using Suckling Hamsters Inoculated With Equine Herpesvirus 9 (EHV-9): Kinetics of the Virus and Time-Course Pathogenesis of EHV-9–Induced Encephalitis via the Eyes

N. El-Habashi[1], Y. Kato[2], E. EL-Nahass[2,4], H. Fukushi[2], A. Hirata[2], H. Sakai[2], J. Kimura[3], and T. Yanai[2]

Abstract

By using a new member of the neurotropic equine herpesviruses, EHV-9, which induced encephalitis in various species via various routes, an ocular infection model was developed in suckling hamsters. The suckling hamsters were inoculated with EHV-9 via the conjunctival route and were sacrificed after 6, 12, 24, 36, 48, 72, 96, 120, and 144 hours (h) post inoculation (PI). Three horizontal sections of the brains, including the eyes and cranial cavity, were examined histologically to assess the viral kinetics and time-course neuropathological alterations using a panoramic view. At 6 to 24 h PI, there were various degrees of necrosis in the conjunctival epithelial cells, as well as frequent mononuclear cell infiltrations in the lamina propria and the tarsus of the eyelid, and frequent myositis of the eyelid muscles. At 96 h PI, encephalitis was observed in the brainstem at the level of the pons and cerebellum. EHV-9 antigen immunoreactivity was detected in the macrophages circulating in the eyelid and around the fine nerve endings supplying the eyelid, the nerves of the extraocular muscles, and the lacrimal glands from 6 h to 144 h PI. At 96 h PI, the viral antigen immunoreactivity was detected in the brainstem at the level of the pons and cerebellum. These results suggest that EHV-9 invaded the brain via the trigeminal nerve in addition to the abducent, oculomotor, and facial nerves. This conjunctival EHV-9 suckling hamster model may be useful in assessing the neuronal spread of neuropathogenic viruses via the eyes to the brain.

Keywords

EHV-9, eye, suckling hamsters, neuropathogenesis

EHV-9, the newest member of the equine herpesvirus family, is a highly neurotropic herpesvirus first isolated in an outbreak of encephalitis in a herd of Thomson's gazelles (*Gazella thomsoni*) kept at a zoological garden.[13,36] Although the natural host of EHV-9 and the complete host range are still unknown, a member of the equidae is suspected to be a natural host of EHV-9, as domestic horses (*Equus caballus*) inoculated intranasally with EHV-9 exhibited only mild encephalitis and there were no resulting deaths.[34] In addition, a high seroprevalence for EHV-9 without any signs was detected among Burchell's zebras (*Equus burchelli*) in Serengeti National Park in Tanzania.[2]

Recently, it has been reported that the host range of EHV-9 has expanded to include Grevy's zebras (*Equus grevysi*) and polar bears (*Ursus maritimus*),[7,31] as well as a giraffe (*Giraffa camelopardalis reticulata*).[18] EHV-9 is most closely related to the recently re-emergent neurotropic pathogen, EHV-1; however, cleavage by restriction enzymes showed that the DNA fingerprint of EHV-9 is different from that of EHV-1 and other equine herpesviruses.[13]

Emerging EHV-9 infections are of special interest because there is a wide range of susceptible hosts, which includes mice and rats,[13] hamsters,[12] goats,[35] pigs,[26] dogs and cats,[37,38] and common marmosets.[21] Recently, EHV-9 was used to induce encephalitis in hamsters by different routes of inoculation, including the oral (25%), peritoneal (25%), and ocular (75%) routes; however, the intravenous route failed to induce

[1] Department of Veterinary Pathology, Faculty of Veterinary Medicine, Kafr El-Sheikh University, Kafr El-Sheikh, Egypt
[2] Department of Veterinary Pathology and Microbiology, Faculty of Applied Biological Science, Gifu University, Gifu, Japan
[3] Department of Veterinary Pathology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt.
[4] Department of Veterinary Anatomy, College of Veterinary Medicine, Seoul National University, Seoul, Korea

Corresponding Author:
Tokuma Yanai, Department of Veterinary Pathology and Microbiology, Faculty of Applied Biological Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan.
Email: yanai@gifu-u.ac.jp

encephalitis and there were discrepancies in the distribution of EHV-9 antigen immunoreactive cells in the location and severity of the cerebral lesions. This suggests the possibility of transmission of the virus through regional nerves (the non-olfactory route), after initial propagation at the site of viral entry.[9] The exact pathway through which the virus travels from the eye, which proved to have a high incidence of infection (75% of the animals became infected) in comparison to the other routes, to the brain is still unclear.

Thus, the aim of the present study is to elucidate the pathogenesis of EHV-9 ocular-induced encephalitis. Ocular transmission of viruses to the central nervous system (CNS) has been suspected in many viral diseases such as Cercopithecine herpes virus 1 (B-virus) in humans[5] and herpes simplex virus in children with labial vesicles after rubbing of the eye.[14] Fatal infection by B-virus in humans via ocular exposure from biological fluid from macaque monkeys has been reported.[5] A number of viruses have been studied following intraocular inoculation. In general, intraocular inoculation has been found to be effective in inducing CNS infection, with or without inducing specific ocular pathological lesions. Many viruses, including poliomyelitis virus,[10,22] rabies virus,[29] vesicular stomatitis virus,[30] fox encephalitis virus,[15] and equine encephalomyelitis,[20] successfully induced brain infection following intraocular inoculation. The ocular route may be important in the transmission of many viruses in the laboratory as well as in the field.

In the present study, we describe the use of a suckling hamster model to assess primary infection in the eye, followed by the spread of the virus through neuronal routes to the brain. The suckling hamsters were useful in assessing viral kinetics and pathogenesis because panoramic horizontal views of the head, including the eye, orbital cavity, nasal cavity, cranial nerves, and brain, are very similar to MRI (magnetic resonance imaging) in human radiography. In addition, the eyes were still closed at this age in the suckling hamsters, which enabled the precise delivery of the exact inocula to an enclosed site in the conjunctival sac between the eye and eyelid, which is not available in adult animals with open eyes. Suckling hamsters have been used in the past to elucidate the pathogenesis of various viruses, including the mumps virus, via intracerebral inoculation,[23,33] and recently, the suckling hamster model was used to study the spread of EHV-9 from the nasal cavity to the brain by nasal inoculation.[8]

Materials and Methods

Animals

Nine pregnant Syrian hamsters (*Mesocricetus auratus*) dams at gestational day 10 were purchased from a commercial breeder (SLC Inc., Hamamatsu, Japan). The animals were housed in an isolated biohazard cabinet and were fed a basal pellet diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and bottled water ad libitum until they gave birth, after which they were left to rear the pups until the end of the experiment. This experiment was conducted in accordance with the pertinent laws and regulations on the treatment and use of laboratory animals. The experiment protocol was approved by the Animal Experiment Committee for Animal Welfare in the Faculty of Applied Biological Science at Gifu University.

Inoculation of virus. Twenty-nine 6-day-old suckling hamsters were bilaterally inoculated via the conjunctival route with 10 μL (10^6 PFU) of EHV-9 virus solution. The eyelid was grasped, and virus solution was injected into the conjunctival sac between the eyelid and eye (the eyes are still closed in animals at this age). Three each of the inoculated animals were sacrificed at 6, 12, 24, 36, 48, 72, 96, 120, and 144 h postinoculation (PI), respectively, or when they were moribund (one animal after 96 hr and another after 120 hr PI). Five uninfected animals were kept as a control group and were inoculated in the conjunctival sac with a sham inoculum that consisted of sterile cell culture medium (MEM). The animals were checked for clinical signs at least twice daily, in the morning and afternoon. Two inoculated animals died after 6 h PI and their data were excluded from the experiment as we believed that the animals died as a result of the inoculation procedure.

Necropsy, histopathology, and immunohistochemistry. After necropsy, the heads were separated from the bodies and were fixed in 10% neutralized buffered formalin. The fixed heads were sectioned on the horizontal plane at two levels (the first section extending cranially from the supraorbital area passing through the cerebral cortex, the second section extending cranially through the eye and brain; from the second section, ventral [Fig. 1a] and dorsal [Fig. 1b] sections were obtained) to obtain the connection between the eye and brain at the same section and were then dehydrated and embedded in paraffin wax by routine methods. They were then sectioned at 3 μm, stained with hematoxylin and eosin (HE), and examined by light microscopy. The bones, including the skulls, of the suckling hamsters were soft enough to prepare histological slides with good quality without decalcification.

Paraffin-embedded sections of the head, including the brain and eyes, were immunolabeled with EHV-9 rabbit antiserum by the avidin-biotin-complex (ABC) immunoperoxidase method, as described previously,[38] with ABC kits (Vector Laboratories, Burlingame, CA, USA). The primary antibody was EHV-9 antiserum (1:800, Veterinary Microbiology Laboratory at Gifu University) followed by application of a secondary antibody (biotinylated anti-rabbit IgG, DAKO Cytomation, USA) with Liquid DAB Substrate Chromogen System (DAKO Cytomation, USA) used as the chromogen and hematoxylin counterstain. Tissue sections from confirmed EHV-9–infected hamsters were used as positive control samples, and sera from a nonimmunized rabbit and goat were used as a negative control treatment on positive control specimens.

Results

Clinical Findings

The inoculated hamsters showed various degrees of clinical signs, including depression and uncoordinated movements,

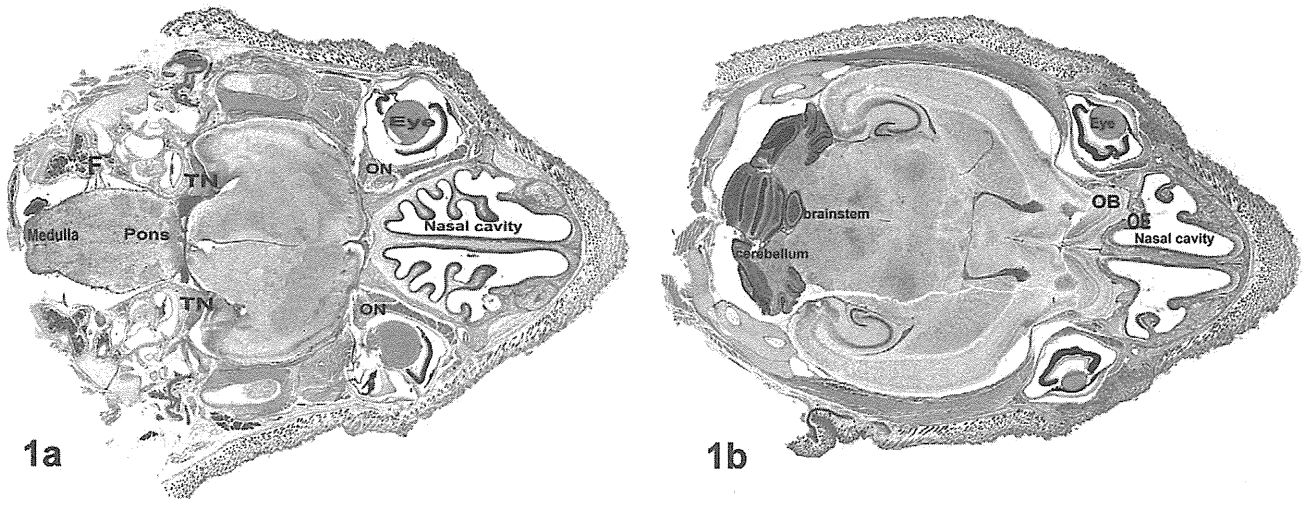

Figure 1. a. Hamster, brain, horizontal section showing the head from the eye to the whole brain from the ventral view; TN, trigeminal nerve; ON, optic nerve; F, facial nerve. Hematoxylin and eosin (HE). b. Hamster, brain, horizontal section showing the head from the eye to the whole brain from the dorsal view at the level of the forebrain, brainstem, and nasal cavity; OE, olfactory epithelium; OB, olfactory bulb. HE.

Table 1. Histopathological Changes Induced in the Eye and Brain in EHV-9 Inoculated Animals.[a]

		Hours Post Inoculation (*n*)[b]								
Tissue	Lesion	6 (3)	12 (3)	24 (3)	36 (3)	48 (3)	72 (3)	96 (3)	120 (3)	144 (3)
Conjunctiva	Necrosis (x)[c]	+ (1)	+ (2)	++ (2)	++ (2)	—	—	—	—	—
	Infiltration of inflammatory cells	+ (2)	++ (3)	+++ (3)	+++ (3)	++ (2)	+ (2)	—	—	—
Lacrimal gland	Infiltration	—	++ (1)	++ (2)	++ (2)	+ (2)	+ (1)	+ (1)	+ (1)	—
Eye muscles	Myositis	—	—	+ (1)	+ (2)	++ (2)	++ (2)	+ (1)	—	—
Brain	Brainstem encephalitis	—	—	—	—	—	—	+ (2)	++ (2)	+++ (3)
	Extended encephalitis	—	—	—	—	—	+ (1)	—	+ (2)	++ (3)

[a]Histopathologic score: —, none; +, slight; ++, moderate; +++, severe.
[b]*n*, number of sacrificed animals at this hour post inoculation.
[c]x, number of animals showed the pathological changes.

starting at 96 h PI. By 120 h PI, all of the animals exhibited severely uncoordinated movement, as well as paralysis, sometimes unilaterally. The animals were unable to crawl normally, were dysphagic, became depressed, and eventually died from the neurologic disorder.

Gross observation and histopathology. Grossly, there were no apparent abnormalities in the organs or tissues of the inoculated animals. Horizontal sectioning of the head in the suckling hamsters made it easy to view the orbital cavity, including the eye with all of its compartments, and most of the cranial nerves connections to the brain and brain itself were visible in a histological section (Figs. 1a, 1b). No microscopic abnormalities were noted in the five uninoculated control mice at any interval of the experiment. Histopathological alterations in the eye and brain in animals inoculated with EHV-9 via the conjunctival route are summarized in Table 1 and as follows:

At 6 h PI: Single cell necrosis in the conjunctival mucosal epithelial cells was observed, along with a few infiltrations predominantly neutrophils, macrophages, and few lymphocytes, in between and associated with the conjunctival mucosal epithelial cells and often free in the conjunctival sac in comparison to control animals.

At 12 h PI: There were variable but increased numbers of neutrophils, macrophages, and lymphocytes in the lamina propria and tarsus of the eyelid, which sometimes extended to the connective tissue of the cutaneous part of the eyelid, along with myocyte necrosis within the eyelid muscles, associated with increased numbers of macrophages and few neutrophils and lymphocytes (Fig. 2). In addition, there were varying increases in macrophages and lymphocytes in the lacrimal glands, as well as a slight focal necrosis and desquamation of the epithelial cells of conjunctival mucosa. In some cases, the inflammatory reaction extended from the subcutis of the eyelid to the skin in the face.

At 24 h PI: There were multifocal necrosis and desquamation of the conjunctival mucosal epithelium (Fig. 3), together with marked increases in neutrophils, macrophages, and lymphocytes in the eyelid, myositis of extraocular muscles characterized by

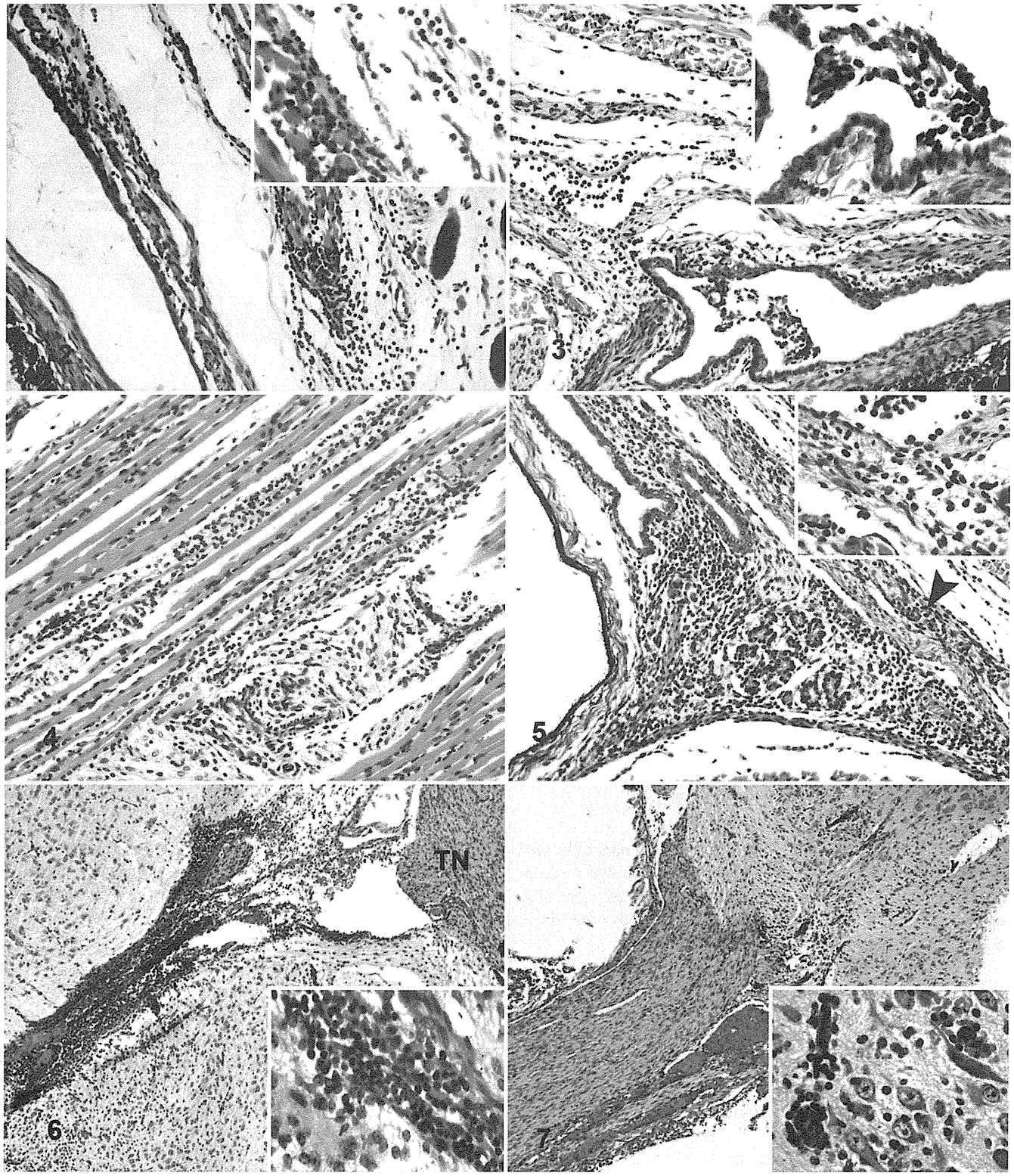

Figure 2. Suckling hamster, eyelid; 12 h post inoculation, there is infiltrations of neutrophils, macrophages, and lymphocytes in the epithelium, lamina propria and muscles of the eyelid, in addition to necrosis of the eyelid muscle. Inset: Higher magnification showing myositis (macrophages and a few lymphocytes and neutrophils) myocyte necrosis and myophagia. Hematoxylin and eosin. **Figure 3.** Suckling hamster, eyelid; 24 h post inoculation, there is conjunctival epithelial cell necrosis and inflammatory infiltrates of neutrophils, macrophages, and lymphocytes, in the lamina propria, tarsus. Inset: Higher magnification showing necrosis and sloughing of the conjunctival epithelial cells. Hematoxylin and eosin.

moderate increases in macrophages and lymphocytes (Fig. 4). Vasculitis, perivasculitis, and neuritis of the nerves supplying these muscles were observed in few animals and moderate infiltrations of the lacrimal glands with macrophages and lymphocytes were also observed.

At 36h PI: There were marked increases in macrophages, lymphocytes, and few neutrophils in the eyelid (Fig. 5) and within the terminal endings supplying the eyelid, with associated edema of nerve fibers and disruption of nerve axons.

At 48 h PI: There was mild meningitis in the form of mild focal or diffuse lymphocytes infiltration together with macrophages and few neutrophils and hemorrhages at the level of the entrance of the trigeminal sensory nerve to the brainstem, as well as mild neuritis of the trigeminal sensory nerve characterized by few lymphocytic cell infiltrations and vasculitis. The trigeminal and palatine ganglion showed moderate neuronal necrosis and glial reactions consisting of neuronophagia to the ganglion cells. In addition, a few neuroglial cells and lymphocytes had infiltrated the area around the ganglion cells. Very mild increases of macrophages and lymphocytes in the eyelid as well as myositis of the extraocular muscles were observed.

At 72 h PI: One animal with neuronal signs had meningoencephalitis extending from the olfactory bulb to the brainstem, which consisted of neuronal necrosis, perivascular aggregates consisting of lymphocytes, macrophages, and few neutrophils, gliosis, intranuclear inclusion bodies, and diffuse lymphocytic infiltrates together with few macrophages and vasculitis in the meninges. In addition, the olfactory epithelium in this animal showed severe and wide erosion in the mucosa, together with significant purulent inflammation. Trigeminal ganglionitis was also observed. However, the other animals had only meningitis extending to the Virchow-Robin space characterized by infiltration of lymphocytes and neutorphils and haemorrhages (Fig. 6) at the level of the brainstem at the entrance of the trigeminal sensory nerve. Myositis of the extraocular muscles was observed in some cases, in addition to mild neuritis of the nerves supplying those muscles.

At 96 h PI: The brainstem at the level of the pons, especially at the entrance of the sensory branch of the trigeminal nerve to the brain (Fig. 7), exhibited encephalitis consisting of neuronal degeneration and necrosis, perivascular cuffing of lymphocytes, macrophages, and few neutrophils and gliosis, which extended to the trigeminal sensory nucleus.

At 120 h PI: The encephalitis was observed in the pons and medulla oblongata, including the entrance of the sensory branch of the trigeminal nerve, abducent, and the facial nerves.

At 144 h PI: In addition to the pons and medulla oblongata, the encephalitis was also observed in the frontal and temporal lobes of the cerebral cortex in the brain as well as the cervical part of the spinal cord.

Immunohistochemistry. Immunohistochemical staining with EHV-9 antibody was used to determine the viral kinetics of transmission from the eye to the brain. The immunohistochemical detection of EHV-9 in the eye and the brain is summarized as follows:

At 6 to 12 h PI: Occasional nuclear immunoreactivity was observed in the epithelial cells of the conjunctival mucosa (Fig. 8), as well as cytoplasmic and membrane-associated immunoreactivity of most likely infiltrating macrophages and possibly local dendritic cells in the lamina propria and tarsus of the eyelid. Other cells of the eyelid and eyes showed no immunoreactivity.

At 24 h PI: There was frequent immunoreactivity in the macrophages, especially around the fine nerve endings of the eyelid and the macrophages infiltrating the extraocular muscles of the eye as well as those infiltrating the lacrimal glands.

At 36 to 48 h PI: There was frequent and more intense immunoreactivity in the macrophages in the eyelid around the nerve endings (Fig. 9), the macrophages that had infiltrated the lacrimal glands (Fig. 10), the muscles of the eyes, and the area around the nerves supplying muscles before the entrance to the cranial cavity. No immunoreactivity was observed in the brain.

At 72 h PI: In addition to macrophages freely migrating through the eyelid and around the eye, lacrimal glands, and eye muscles, there was cytoplasmic and membrane-associated EHV-9 immunoreactivity in olfactory epithelium and trigeminal ganglia, nuclear and cytoplasmic immunoreactivity in neurons in olfactory bulb, brainstem, and cytoplasmic immunoreactivity in inflammatory cells, and nerve fibers at the trigeminal nerve entrance to the brainstem (Fig. 11) in the case that showed nervous signs. However, no immunoreactivity was observed in the brain in other cases.

At 96 h PI: EHV-9 antigen immunoreactivity was found only in neurons and few inflammatory cells in the brainstem at the level of the pons and cerebellum, close to the entrance of the sensory branch of the trigeminal nerve; however, the olfactory bulb and other areas of the brain showed no immunoreactivity to EHV-9 antigen. In addition, the macrophages present within the eyelid and the eye muscles and lacrimal glands showed immunoreactivity to EHV-9 antigen, but it was less intense than those in

Figure 4 (continued). Figure 4. Suckling hamster, extraocular muscle; 24 h post inoculation, there is myositis characterized by macrophages and lymphocytes infiltration and myocyte necrosis. Hematoxylin and eosin. **Figure 5.** Suckling hamster, eyelid; 36 h post inoculation, there is severe infiltration of inflammatory cells mostly of lymphocytes, as well as neuritis of the terminal nerve endings (arrow) supplying the eyelid. Higher magnification showing neuritis of the terminal nerve endings characterized by infiltration mostly of lymphocytes and disruption of the nerve axon. Hematoxylin and eosin. **Figure 6.** Suckling hamster, brain; 72 h post inoculation, trigeminal sensory nerve (TN) meningoencephalitis and perineuritis, with hemorrhage. Inset: Higher magnification showed infiltration mainly of lymphocytes in the meninges. Hematoxylin and eosin. **Figure 7.** Suckling hamster, brain; 96 h post inoculation, the entrance of the sensory branch of the trigeminal nerve showed mild neuritis and perineuritis. Inset: Higher magnification of the pons showed encephalitis consisting of neuronal degeneration and necrosis, perivascular cuffing, and gliosis. Hematoxylin and eosin.

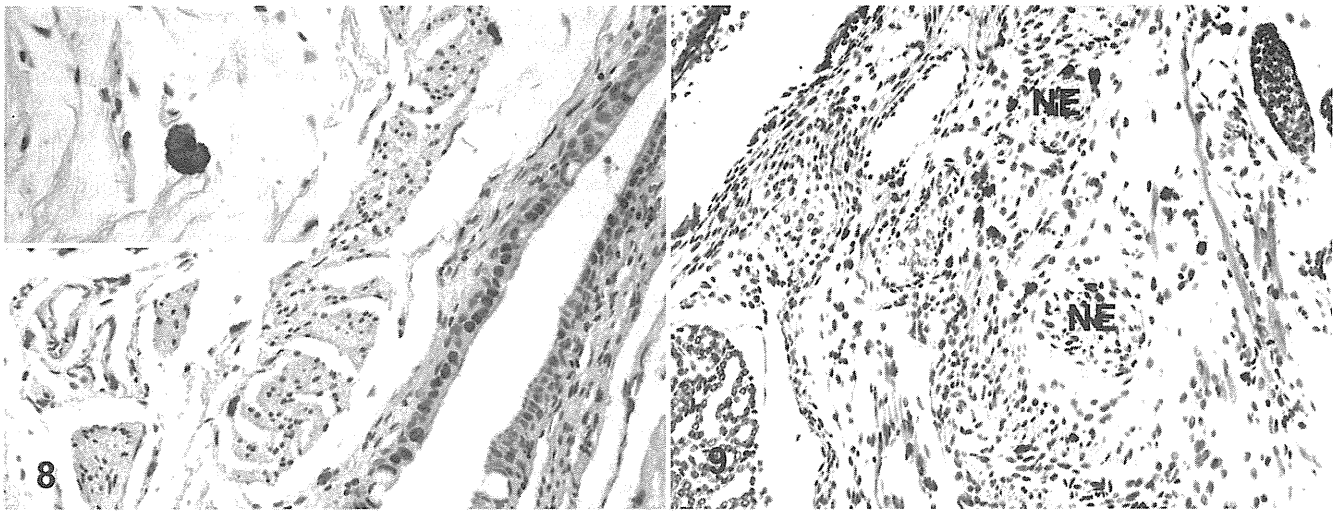

Figure 8. Suckling hamster, eye; immunolabelling with EHV-9 antibody, the conjunctival epithelial cells exhibited a positive immunoractivity. Inset: Higher magnification showed immunoreactive macrophage. ABC method, counterstained with Mayer's hematoxylin. **Figure 9.** Suckling hamster, eye; immunolabelling with EHV-9 antibody. At 36 to 48 h post inoculation, positive reaction was observed in most of the macrophages circulating around the nerve endings (NE). ABC method, counterstained with Mayer's hematoxylin. **Figure 10.** Suckling hamster, eye; immunolabelling with EHV-9 antibody. At 36 to 48 h post inoculation, positive reaction was observed in some of the macrophages infiltrating the lacrimal gland in the eyelid. ABC method, counterstained with Mayer's hematoxylin. **Figure 11.** Suckling hamster, brain; immunolabelling with EHV-9 antibody. At 72 h post inoculation, viral antigen was present in some of the inflammatory cells of the trigeminal sensory nerve root. ABC method, counterstained with Mayer's hematoxylin.

previous cases. The neurons of trigeminal ganglia showed weak immunoreactivity in their cytoplasm.

At 120 to 144 h PI: There was immunoreactivity in the neurons and some inflammatory cells in the medulla oblongata, with specific intensity in the midline and close to the entrance of the abducent nerve, as well as the pons and cerebellum. In addition, the viral antigen immunoreactivity was detected in some neurons in the frontal and temporal lobes of the cerebral cortex. EHV-9 was detected in the cervical part of the spinal cord, in the neurons and inflammatory cells in areas showing myelitis.

The distributions of the viral antigen immunoreactivity in the horizontal sections of the brain after 96 h and 144 h PI are depicted in Figures 12a and 12b, respectively. At 96 h, EHV-9 antigen immunoreactivity was detected only in the brainstem at the level of the pons close to the entrance of the sensory branch of the trigeminal nerve; however, at 144 h PI EHV-9 antigen immunoreactivity was detected in the medulla oblongata, close to the entrance of the abducent nerve, as well as the pons and extended to frontal and temporal lobes of the cerebral cortex.

Discussion

EHV-9, the newly emerging neurotropic herpesvirus, has been studied with respect to pathogenesis and route of brain infection including the nasal, ocular, peritoneal, oral, and

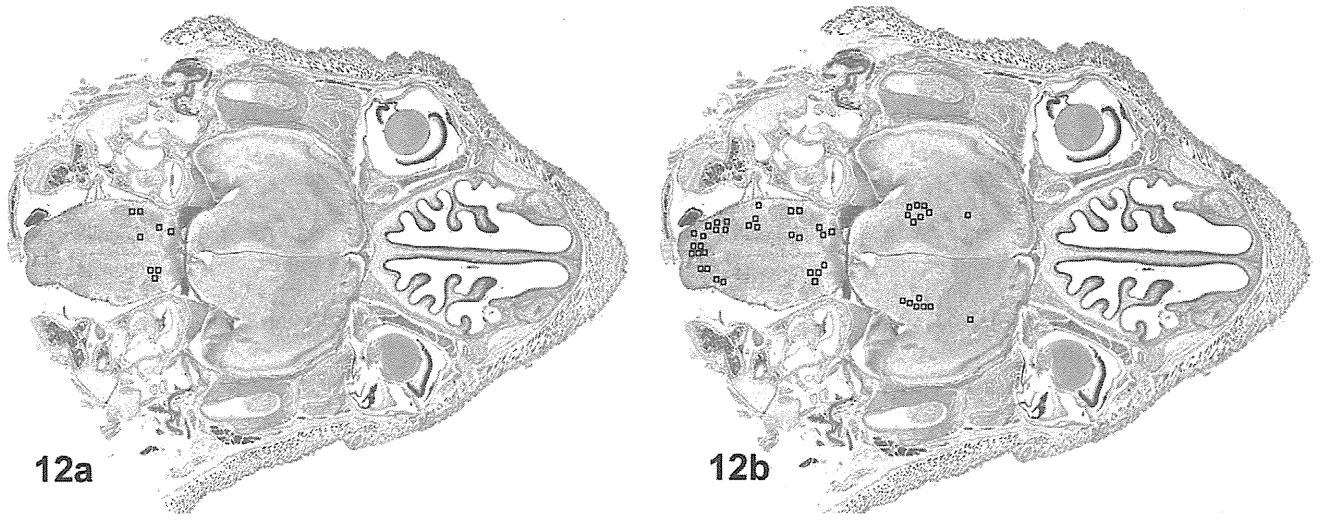

Figure 12. a. Suckling hamster, brain, distribution of EHV-9 antigen immunoractivity. At 96 h post inoculation (PI), viral antigen was detected in the pons. b. Suckling hamster, brain, distribution of EHV-9 antigen immunoractivity. At 144 h PI, viral antigen was found in the pons, cerebellum, medulla oblongata, and the frontal and temporal lobes of the cerebral cortex.

intravenous routes. The nasal and ocular routes show higher incidences of infection (100% and 75% of animals became infected, respectively) in comparison to the other routes.[9] The ocular route for the transmission of neuropathogenic viruses is gaining in importance in the laboratory as well as in the field. Fatal infections with Cercopithecine herpes virus 1 (B-virus) via ocular splashes with biological fluid from macaque monkeys without percutaneous injury have been reported in humans,[4,5] and in this case, conjunctivitis developed 10 days later after exposure, and the victim then died from B-virus–induced encephalomyelitis 6 weeks later. Possible infection in the CNS following ocular inoculation was reported with respect to many viruses, such as EHV-9 in hamsters,[9] poliomyelitis virus,[10,22] rabies virus,[29] vesicular stomatitis virus,[30] fox encephalitis virus,[15] and equine encephalomyelitis.[20] However, the detailed kinetics and pathogenesis in terms of access to the CNS are still unclear because of difficulties in tracing the entrance of the cranial nerves to the brain through the cranial bone. The suckling hamster model following conjunctival inoculation with EHV-9 was developed to assess the kinetics and pathogenesis of EHV-9 using a panoramic view that includes the eye, olfactory epithelium, olfactory nerve, and optic nerve as well as other cranial nerves and the brain by using horizontal sections. Horizontal sections at two levels were prepared in the present study to assure easy tracking from the viral inoculation site of the eye to the main affected site of the brain. Based on histology and immunohistochemistry, conjunctival inoculation with EHV-9 first results in conjunctivitis characterized by conjunctival epithelial cell necrosis, mononuclear cell infiltrations in the lamina propria of the eyelid, as well as myositis of the eyelid muscles and infiltration of lacrimal glands, which may be an essential stage for propagation of the virus in the entry site for further travel to the CNS. At the early stage of infection (6 hr PI), the conjunctival epithelial cell as well as macrophages exhibited immunoreactivity to EHV-9 antigen, especially around the fine nerve endings, and this extended later to the eyelid muscles, lacrimal and extraocular muscles, as well as the nerves supplying those muscles. At the initial stage of infection, the macrophages migrating from surrounding tissue may play a very important role in the propagation and transmission of EHV-9 to the nerves of the eyes. Previously, the importance of macrophages in the pathogenesis of virus infections was reviewed by Mires.[24] Blood clearance studies suggested that most viruses are taken up by macrophages in the same way as other colloidal materials.[3,24] Since macrophages move freely through tissues, it has also been suggested that they play a primary role in the dissemination of viruses.[11] Therefore, the susceptibility or resistance to viral diseases could depend in large part on the macrophage-virus interaction. In an in vitro study on macrophages in suckling mice, herpesvirus was shown to have spread to adjacent cells of various types by macrophages.[17] The migrating macrophages may pick up the EHV-9 and transport it to the nerve endings supplying the eyelid and eye, including the ophthalmic branch of the trigeminal nerve and the abducent, oculomotor, and facial nerves. EHV-9 may then travel by way of these nerves to the brainstem causing encephalitis at the level of the pons and cerebellum. CNS lesions in these animals were restricted to the pons, medulla oblongata, and cerebellum, suggesting viral transport through the trigeminal, abducent, and facial nerves. A similar transneural passage has been described in porcine Pseudorabies virus (PRV) infection where virus gained access to the brain via the trigeminal and oculomotor nerves.[32] In cases of herpes simplex virus infection in rabbits, the inoculated virus was passed serially onto the scarified cornea, producing fatal encephalitis, where the virus did not enter the eye after inoculation onto the cornea, but passed to the conjunctiva and thereafter to the brain via the

sensory branches of the trigeminal nerve.[14] Keeble et al. described lesions of natural B-virus infection in monkeys where CNS lesions were minimal and consisted of localized involvement of the pons and medulla oblongata.[19] In addition there were glial and lymphocytic infiltrations around the nerve roots of the trigeminal and facial nerves, in the nucleus and tract of the descending branch of the trigeminal nerve and in the solitary tract.[19] Encephalitis restricted to the brainstem was reported previously in cases of human herpes simplex virus 1 infection[16,25] and herpes simplex virus 2 infection.[6,28]

At the final stage EHV-9 virus expanded rostrally to the cerebrum as well as caudally to the spinal cord. There were some discrepancies in severity and distribution of the lesions. In some cases, the encephalitis was restricted to one-half of the brain, or there was bilateral involvement. The ocular EHV-9 suckling hamster model may be useful in pathogenesis studies of neurotropic virus such as B-Virus, especially those concerned with how virus gains access to the brain.

The optic nerve is considered to be part of the central nervous system, which is completely encased by three layers of meninges and is protected from contact with macrophages carrying the virus. With some viruses, such as the lymphocytic choriomeningitis virus (LCM), multiplication of the virus in the eyes does not induce ocular pathological changes following intraocular inoculation except mild transitory hyperemia;[27] however, in the present study marked conjunctivitis was noted. In our previous work with EHV-9 after nasal inoculation, we found that the virus traveled through the olfactory nerve after initial propagation in the olfactory receptor neurons, and that area of the brain that was affected earliest and to the greatest degree was the olfactory bulb;[8] however, in the present study, the pons and cerebellum were affected earliest.

There is still a possibility of other routes of transmission of EHV-9 after conjunctival inoculation, via the oral or nasal routes, because the eyes and the oral and nasal cavities are connected by means of the lacrimal (tear) duct. After conjunctival inoculation, (1) that virus may secondarily infect the olfactory nerve via the infection of the lacrimal gland, and (2) virus may also spread to the nasopharynx with the tear film via the nasolacrimal duct, providing an alternate method of infecting the olfactory nerve and accounting for the rhinitis noted in one animal with possibly dominant and earliest lesions in the olfactory bulb.

The suckling hamster model, using horizontal sections of the head following conjunctival inoculation, is very useful in elucidating the mechanism of EHV-9 transmission from the eye to the brain. Following conjunctival inoculation, EHV-9 appears to result in local conjunctivitis and infection of local macrophages, which transport the virus to local fine nerve endings; because of this, EHV-9 is able to invade the brain via the trigeminal, abducent, oculomotor, and facial nerves, inducing meningitis, encephalitis, and myelitis.

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Short communication

Molecular characterization of *Echinococcus granulosus* in Egyptian donkeys

Shawky Mohamed Aboelhadid[a,*], Khaled Mohamed El-Dakhly[a], Tokuma Yanai[b], Hideto Fukushi[c], Kamel Mohamed Hassanin[d]

[a] *Department of Parasitology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt*
[b] *Department of Veterinary Pathology, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu, Japan*
[c] *Department of Microbiology, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu, Japan*
[d] *Department of Biochemistry, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt*

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ABSTRACT

The present study was carried out during the period from August 2009 to July 2010. The purpose of this study was to identify the genotype of donkeys' echinococcosis in one of the Egyptian governorates. On post-mortem inspection taking place at the zoo of Beni-Suef, Egypt, a total number of 145 donkeys were examined for the presence of hydatid cysts. Ten of these donkeys were found to be infected by hydatid cysts; and location, number and fertility of cysts found were determined. The liver was the predominant site of infection. Molecular identification of these cystic echinococcosis isolates, based on PCR amplification and the sequence of both mitochondrial ND1 and CO1, revealed that they belonged to *Echinococcus equinus* (G4 genotype). An alignment of ND1 and CO1 partial nucleotide sequences with G4 partial nucleotide sequences revealed replacement of G at position 105 with A and replacement of A at position 276 with G respectively. It can be concluded that the donkeys involved in this study were harboring *E. equinus*. For the first time in Egypt, the present work allowed us to record the presence of the *E. equinus* with the molecular tools, and to report new information on the epidemiological status of this parasite in Egypt.

1. Introduction

Hydatid disease is a zoonotic disease known to spread worldwide. It is caused by larval stages (metacestodes) of tape worm parasite of genus *Echinococcus* that infect different animal species (Rausch, 1995). These domestic animals include sheep, goats, cattle, swines, buffalos, horses, and camels (Bryan and Schantz, 1989). Human beings may also serve as dead-end hosts (Binhazim et al., 1992). *E. granulosus sensu lato* shows intraspecific variation in relation to host, specificity, epidemiology, morphology, developmental biology, biochemistry and genetics (Thompson and McManus, 2002). Based on the genetic characterization which is mostly dependent on the homology of the sequence of the two mitochondrial genes; cytochrome C oxidase subunit 1 (CO1) and reduced nicotinamide adenine dinucleotide subunit 1 (ND1), ten different genotypes, among which G4 (horse strain) have been formerly characterized (McManus, 2002; Lavikainen et al., 2003; Snábel et al., 2009). Recent taxonomic classification suggested the presence of five valid species within the *E. granulosus* species complex. These are named *E. granulosus* s.s. (G1–G3 genotypes), *E. equinus* (G4 genotype), *E. ortleppi* (G5 genotype) and *E. canadensis* (G6–G10 genotypes) and *E. felidis* (Thompson and McManus, 2002; Nakao et al., 2010; Hüttner et al., 2008).

Equine hydatidosis, especially that of donkeys, has been studied in some countries of The Middle East; Lebanon and Jordan (Thompson and McManus, 2002). *E. equinus*

* Corresponding author. Fax: +20 2 82 2327982; mobile: +20 2 01008525674.
E-mail address: Drshawky2001@yahoo.com (S.M. Aboelhadid).

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has been reported from the United Kingdom, Switzerland, Belgium, Italy, Syria and South Africa (Thompson and Lymbery, 1990; Varcasia et al., 2008).

E. equinus (formerly *E. granulosus* G4 or horse strain) appears to be a specific parasite of equids (Jenkins et al., 2005; Nakao et al., 2007; Romig et al., 2006; Thompson, 2008; Saarma et al., 2009; Blutke et al., 2010). Varcasia et al. (2008) identified two species of genus *Echinococcus* infecting horses in Italy; *E. granulosus* s.s. (old G1, sheep strain) and *E. equinus* (old G4 or horse strain). Scant information about this parasite in donkeys is available (Thompson and McManus, 2002). A study in Giza, Egypt, showed the presence of unspecified *Echinococcus* sp. in donkeys (Haridy et al., 2008). However, one of the most recent studies on RFLP patterns of CE isolates from donkeys in Egypt failed to allocate the isolates to any described strain or species (Taha, 2012). Our present work aimed to identify the genotypes of hydatid cysts isolated from Egyptian donkeys, and this was planned to be done by sequencing their PCR-amplified two mitochondrial genes; cytochrome C oxidase subunit 1 (CO1) and NADH dehydrogenase subunit 1 (ND1).

2. Materials and methods

2.1. Parasite samples

This study included 145 donkeys (over five years of age) of local origin. They were raised in different cities of Beni-Suef governorate. Beni-Suef, an Egyptian governorate, located 120 km south of Cairo. The donkeys were brought to Beni-Suef zoo for feeding lions. They were examined for the presence of hydatid cysts on post-mortem inspection at the zoo during the period from August 2009 to July 2010. Examination of all internal organs was also done by using palpation and incision for the detection of hydatid cysts. Intact hydatid cysts, isolated from the infected animals, were put separately in the polythene bags containing ice and brought to Beni-Suef Veterinary College for further processing.

Hydatid fluid was aspirated after washing the cyst with distilled water twice. It was further subjected to centrifugation at 5000 rpm for 5 min, and the sediment was examined under the low power objective of a compound microscope to observe the protoscoleces. Germinal layer (sterile cysts) and protoscoleces (fertile cysts) were randomly collected from the infected animals, and only one cyst from each infected animal was subjected to molecular characterization to assign the status of a single isolate. The material was frozen at −20 °C until further use.

2.2. DNA extraction

DNA was extracted from the germinal layers and/or protoscoleces using the GeneiUltrapure™ Mammalian Genomic DNA Purification Tissue Kit (Bangalore Genei); and according to manufacturer's instructions, the eluted DNA samples were subjected to dryness by putting them in oven at 55 °C for 3 h. The eluted DNA samples were transferred to Japan by air to perform the PCR amplification and DNA sequencing.

2.3. PCR amplification

Two target sequences of the mitochondrial DNA coding for CO1 and ND1 were PCR-amplified by using Takara Ex Taq kit (Takara Biomedicals, Otsu, Japan).

Two conserved primers, JB3 (forward): 5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′ and JB4.5 (reverse): 5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′ (Busi et al., 2007), were used to amplify the mtDNA region corresponding to the part of the CO1 gene.

Two conserved primers, JB11 (forward): 5′-AGA TTC GTA AGG GGC CTA ATA-3′ and JB12 (reverse) 5′-ACC ACT AAC TAA TTC ACT TTC-3′ (Bowles and McManus, 1993; Bowles et al., 1994), were used to amplify the mtDNA region corresponding to the ND1 gene.

The amplification reactions were carried out in a PCR thermal cycler Dice (Takara, Japan); and it was stained with ethidium bromide and photographed. The corresponding amplicons were checked on 1% agarose gel in a Mupid-2× (Advance, Japan).

2.4. DNA sequences analysis

The amplified PCR-products were cut from agarose gels and purified by using GeneiPure™ Quick PCR Purification Kit (Bangalore Genei) according to manufacturer's instructions. The purified PCR products were subjected to automatic DNA sequencing using Rikakan DNA sequencer (Rikakan, Japan). The DNA sequencing was done in both directions by using the forward and the reverse primers for both CO1 and ND1 mitochondrial genes. Nucleotide sequences were first analyzed and edited to check the electropherograms quality by using the software program Finch TV v 1.4.0 (Geospira Inc.©). Using the Basic Local Alignment *Search* Tool "BLAST" search blast.ncbi.nlm.nih.gov, the obtained DNA sequences were subsequently aligned and compared with verified sequences of *E. granulosus* strains available in the Genbank (Table 1). The phylogenetic tree was constructed by using software program "Mega 4". *Taenia saginata* (NC 009938) was used as an outgroup. Bootstrap analyses were conducted using 1000 replicates. Sequences analyzed in the present study were finally deposited in the Genbank and their accession numbers are listed in Table 1.

3. Results

Out of one hundred and forty-five donkeys slaughtered at Beni-Suef zoo for feeding lions, ten animals were found to be infected with hydatid cysts. The infection rate was 6.89%. The hydatid cysts were present only in the livers of the infected animals or in both livers and lungs. Livers showed cysts in all infected cases, while only three of them harbored cysts in their lungs. In the livers, the number of cysts was ten or more in six cases, 8 in two cases, 5 in one case and 4 cysts in the last one. In the lungs, the number of cysts was sharply lower than in livers; as they showed 1 or 2 cysts in each of the infected lungs. The cysts found in the livers and lungs were divided into three types; fertile, sterile and caseous. The fertility of the cysts was determined by the presence of protoscoleces. In the livers, eight cases

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Table 1
Codes and Genbank accession numbers of specimens and reference sequences used for phylogenetic analysis.

Codes and accession numbers of specimens			Genbank accession number		
	CO1	ND1	Genotype	CO1	ND1
BSU-1	JN191313	JN191320	G1	M84661	AJ237632.1
BSU-2	JN191314	JN191321	G2	M84662	AJ237633.1
BSU-3	JN191315	JN191322	G3	M84663	AJ237634.1
BSU-4	JN191316	JN191323	G4	M84664	AJ237635.1
BSU-5	JN191317	JN191324	G5	M84665	AJ237636.1
BSU-6	JN191318	JN191325	G6	M84666	AJ237637.1
BSU-7	JN191319	JN191326	G7	M84667	AJ237638.1
			G8	DQ144021	AB235848.1

possessed fertile cysts, one case was sterile and the last one was caseous, while the lungs of three-infected cases possessed fertile cysts.

When the purified genomic DNA of the ten isolates of donkeys' echinococcosis was used as template for PCR amplification of both mitochondrial genes, we obtained cytochrome C oxidase 1, and reduced nicotinamide adenine dinucleotide 1, and very intense amplification signals of the expected length (450 bp and 500 bp respectively) from all the analyzed isolates (Fig. 1).

The partial nucleotide sequences (476 bp) of ND1and CO1 (369 bp) of the ten isolates obtained from donkeys were aligned with the reference sequences of the genotypes; G1–G8 using BLAST search. All the examined ten isolates produced sequences having 100% homology to the reference partial nucleotide sequence of the *E. equinus* (old G4, horse strain "AJ237635.1" and "M84664" respectively). Seven of these donkeys cystic echinococcosis ND1 and CO1 gene sequences with the reference sequences of genotypes; G1–G8 of ND1 and CO1 genes (Table 1) were used in designing the phylogenetic trees (Fig. 2) that confirmed the preliminary results obtained by BLAST search; and they also revealed that all isolates belonged to the *E. equinus*. An alignment of ND1 and CO1 partial nucleotide sequences with *E. equinus* partial nucleotide sequences revealed replacement of G at position 105 with A and replacement of A at position 276 with G respectively.

4. Discussion

This work presents the first record of *E. equinus* (old G4 or horse strain) as a cause of cystic echinococcosis in donkeys in Egypt. Necropsy of one hundred and forty-five donkeys revealed that ten of them were found to be infected. The majority of infected donkeys (70%) harbored hydatid cysts in their livers only while the minority (30%) harbored hydatid cysts in both livers and lungs. Some authors obtained a similar pattern of hydatid cysts distribution in donkeys in Jordan (Abo-Shehada, 1988; Mukbel et al., 2000). Varcasia et al. (2008) and Acosta-Jamett et al. (2010) also recorded that equines infected with *E. granulosus*, in Italy and north-central Chile, had the majority of hydatid cysts in their livers. On the contrary, Haridy et al. (2008) found that donkeys infected with cystic echinococcosis in Giza, Egypt had hydatid cysts in their livers only, while Blutke et al. (2010) reported that an infected mare with cystic echinococcosis in Germany had hydatid cysts in its lung only. In the present study, most of the livers' hydatid cysts (85%) were fertile while all lungs' hydatid cysts (100%) were fertile. It is reported that equine cystic echinococcosis can be caused by various *Echinococcus* taxa, but only *E. equinus* is known to develop into fertile cysts (Varcasia et al., 2008; Blutke et al., 2010).

It is well established that the use of PCR amplification and sequencing of both mitochondrial genes; cytochrome

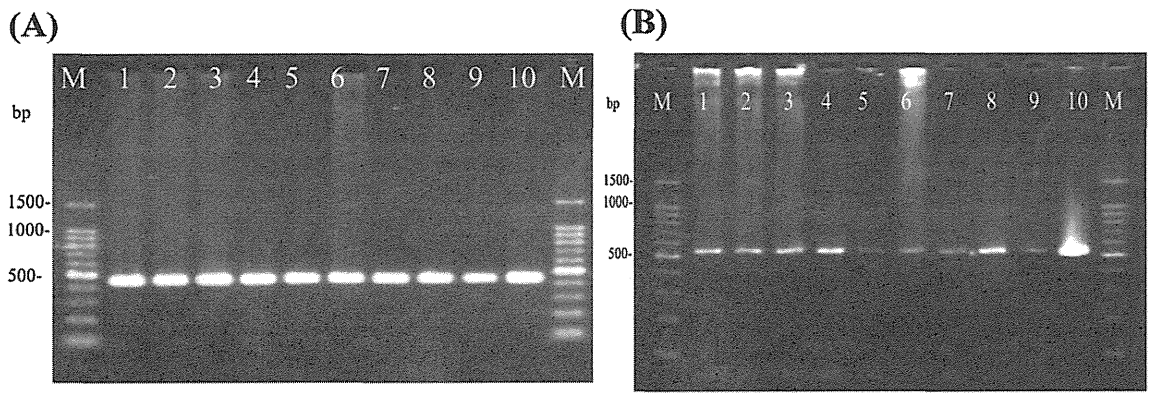

Fig. 1. (A) PCR results of *E. granulosus* template DNA using mitochondrial cytochrome C oxidase subunit 1 gene. Lanes (1–8), lane (10) denote template DNA isolated from protoscoleces of fertile cysts. Lane (9) shows template DNA isolated from germinal layer of sterile cysts. Lane (M), DNA size marker (100-bp DNA ladder). (B) PCR results of *E. granulosus* template DNA using mitochondrial NADH dehydrogenase 1 gene. Lanes (1–8), lane (10) denote template DNA isolated from protoscoleces of fertile cysts. Lane (9) shows template DNA isolated from germinal layer of sterile cysts. Lane (M), DNA size marker (100-bp DNA ladder).

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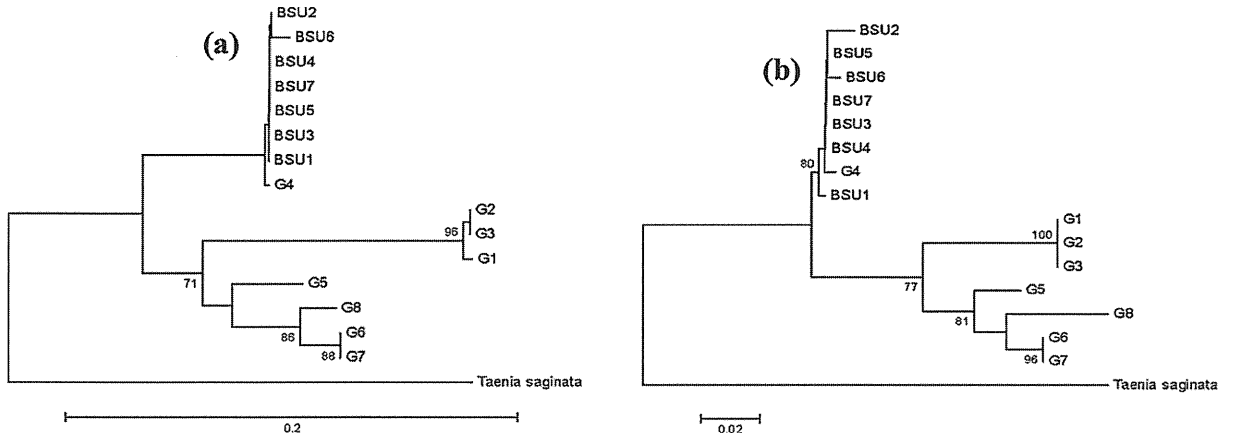

Fig. 2. Genetic relationships between *Echinococcus* isolates from donkeys obtained from Beni-Suef zoo, Egypt and the reference sequences of genotypes G1–G8 as well as *Taenia saginata* as outgroup. The relationships were based on the phylogenetic analysis of concatenated ND1 (a) and CO1 (b) nucleotide sequences of BSU1 to BSU7 using software program "Mega 4".

C oxidase 1 (CO1) and reduced nicotinamide adenine dinucleotide 1 (ND1) is the most common method for genotyping *Echinococcus* isolates (Santivañez et al., 2008; Varcasia et al., 2008; Vural et al., 2008; Sánchez et al., 2010; Beyhan and Umur, 2011; Sharbatkhori et al., 2011). PCR amplification of these two genes in the studied ten isolates produced two bands of the expected length (450 bp and 500 bp respectively). This result is compatible with the results of the previous studies (Beyhan and Umur, 2011; Sharbatkhori et al., 2011).

It is reported that the equids (horses, donkeys and zebras) exclusively act as intermediate hosts for *E. equinus* (Jenkins et al., 2005). For the first time in Egypt, the present study identified that the genotype of the cystic echinococcosis isolates obtained from donkeys in one of the Egyptian governorates as *E. equinus* by partially sequencing of their mitochondrial ND1 (476 bp) and CO1 (369 bp) genes exist. Our results coincided with the findings of Bowles et al. (1992) who found that *E. granulosus* G4 was the causative agent of cystic echinococcosis in horses by sequencing CO1 (366 nucleotides) and ND1 (471 nucleotides) genes. A recent similar study in southern Germany has identified the genotype of hydatid cysts isolates from lungs of mare to be *E. equinus* (Blutke et al., 2010). On the other hand, Varcasia et al. (2008) identified both *E. equinus* (G4 genotype or horse strain) and *E. granulosus* s.s. (old G1, sheep strain) in horses, although cysts of the latter showed aberrant morphological characters and were never fertile.

Donkeys and other equines are infected when grazing pastures are contaminated by feces of dogs and foxes containing the parasite eggs. The donkey and horse act as an intermediate host, in whose livers or lungs fertile hydatid cysts containing protoscoleces are developed (McGorum et al., 1994). This makes the carcasses of these infected equines especially donkeys a major source of infection to the definitive hosts of *E. equinus*, dogs and foxes (Thompson and McManus, 2002). Donkeys infected with cystic echinococcosis may constitute a source of transmitting o infection to dogs and foxes in Egypt. This is because of the unhygienic manners of disposal of the carcasses of perished donkeys. This is usually done by throwing their carcasses near the water canals or cultivable land; the matter that makes them easy to be accessed by stray dogs or foxes. Feeding of donkeys to susceptible carnivores in zoos may also contribute to the transmission of this parasite. Both ways play an important role in the maintenance of *Echinococcus* species life cycle. The hazardous of donkeys' cystic echinococcosis on public health is negligible because it is caused by *E. equinus* which is non pathogenic for human in Egypt. This was reported previously by Thompson (1995), Thompson and McManus (2002) and Romig et al. (2006).

Our genetic characterization of donkeys' echinococcosis strain in Egypt as *E. equinus* will be quite useful in controlling this disease, especially when there is a lack of information about the identity of these parasites, that affect this kind of animals. Finally we recommend post-mortem examination of these donkeys before their introducing as food for wild animals, and we seriously recommend hygienic disposal of the carcasses of these animals and proper condemnation of their affected offal. These precautionary steps will be quite useful in cutting the life cycle of *Echinococcus* species and controlling the disease.

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Key words
Q熱コクシエラ
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人獣共通感染症
ペット

特集　ペットからの感染症　11

Q熱

岸本寿男*　木田浩二*

要旨

Q熱は，Q熱コクシエラ（*Coxiella burnetii*）による人獣共通感染症であり，主に反芻動物やペット等の感染動物の胎盤や排泄物からのエアロゾルによる感染で，非定型肺炎や肝炎，不明熱を起こす．欧米では，しばしば集団発生等もあり，非定型病原体の一つとして一般に認知されている．しかしわが国では，感染症発生動向調査で4類全数報告疾患として報告される数は，年に数例～40例程でほとんどが散発例であり，感染源などまだ実態は不明な点が多い．本稿ではQ熱の概説をし，Q熱の疫学，臨床像，診断，治療，予防について述べ，今後の課題を示す．

Ⅰ Q熱と病原体

Q熱という病名は，「Query fever＝不明熱」に由来する．1935年オーストラリアの屠畜場従業員の間で流行した原因不明の熱性疾患として発見され，のちにリケッチアの一種 *Coxiella burnetii* による感染症であることが明らかになった[1)~3)]．*C. burnetii* は従来リケッチア科コクシエラ属に分類されていたが，最近の遺伝子学的な解析による新たな分類ではレジオネラ目コクシエラ科コクシエラ属に変更された(表1)．

C. burnetii は細胞内でのみ増殖できる偏性細胞内寄生細菌で，人工培地では増殖できない．小桿菌で，その大きさは0.2～0.4×1.0 μmで，一般細菌の球菌の1/2～1/4である．多型性を示し，増殖時の菌の形態には大型菌体（large cell variant：LCV）と小型菌体（small cell variant：SCV）とがある．ともに感染性があり，LCVは浸透圧に対し抵抗性が低いが，SCVは芽胞様構造を示し，熱，乾燥，消毒等に抵抗性のため環境中で長期間安定である．そのため，菌の伝播にダニなどのベクターを必要としない．また，本菌は腸内細菌に似た相変異を起こし，Ⅰ相菌およびⅡ相菌とよばれている．Ⅰ相菌は野外株で菌体表面にリポ多糖（LPS）を保有し，Ⅱ相菌はⅠ相菌を発育鶏卵や培養細胞を用いて長期継代し弱毒化した株でLPSを保有しない．このⅠ相菌およびⅡ相菌が，血清診断でそれぞれ慢性型と急性型の鑑別に重要となる．

Ⅱ 感染様式

本菌は自然界では多くの動物が保菌しており，感染動物の尿，糞，乳汁などに排泄され，環境を汚染する．ヒトへの感染源はおもに家畜や愛玩動物の排泄物であり，これらに汚染された環境中の粉塵やエアロゾルを吸入し感染する．まれにウシやヒツジの未殺菌の乳製品・生

* 岡山県環境保健センター
〒701-0298 岡山県岡山市南区内尾739-1

表1　主なリケッチア性疾患と関連疾患

病原体		病名	流行地	ベクター
目・科	属・種			
リケッチア目・リケッチア科（発疹チフス群）	*Rickettsia prowazekii*	発疹チフス	世界各地	シラミ
	R. typhi	発疹熱	世界各地	ノミ
（紅斑熱群）	*R. japonica*	日本紅斑熱	日本	マダニ
	R. rickettsii	ロッキー山紅斑熱	北・中・南米	マダニ
	R. conorii	ボタン熱（地中海紅斑熱）	地中海沿岸・アフリカ・インド	マダニ
	R. akari	リケッチア痘	北米・アフリカ・ロシア・韓国	小型のダニ
	R. afrikae, R. helvetica, R. honei etc	African tick bite fever etc	世界各地	マダニ
	Orientia tsutsugamushi	つつが虫病	日本（北海道を除く）・アジア各地	ツツガムシ
リケッチア目・アナプラズマ科	*Ehrlichia chaffeensis*	エーリキア症	北中南米・欧州・アフリカ・韓国	マダニ
	Anaplasma phagocytophilum	アナプラズマ症	北中南米・欧州・韓国	マダニ
	Neorikcettsia sennetsu	腺熱	西日本	不明
リゾビア目・バルトネラ科*,**	*Bartonella quintana*	塹壕熱	世界各地	シラミ
	B. henselae	ネコひっかき病	世界各地	—
レジオネラ目・コクシエラ科*	*Coxiella burnetii*	Q熱	世界各地	（マダニ）

*：分子生物学的性状から，従来のリケッチア類から分けられている．
**：人工培地で増殖可能．下線は4類感染症

肉などを摂食し感染することもある（図）．感染動物は不顕性感染で，通常は無症状であるが，妊娠しているウシやヒツジが感染すると，胎盤で爆発的に増殖し流産や死産を起こすこともある．このため，菌を大量に含む胎盤や羊水が原因となったヒトの集団感染が諸外国で多く報告されている．また，ペットからの感染例も報告されており，ヨーロッパでネコの出産や流産が集団発生に関連したとの報告や，本邦でも飼育していたネコとの関連が示唆される例がある．ネコでは大部分が不顕性感染と思われる．ただし，実験感染ネコでは発熱，食欲不振，元気消沈などの症状が発現したとの報告もされている．感染はネコの流産に関係しているが，正常分娩のネコからも病原体は分離されている．一方で，ヒトからヒトへの感染は，罹患者からの

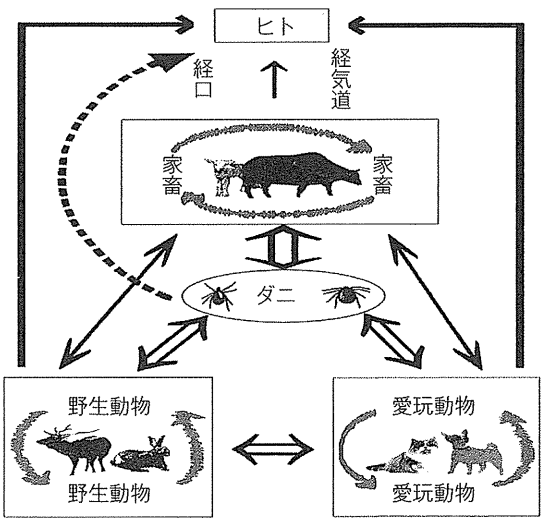

図　環境中の *Coxiella burnetii* の感染様式

輸血等を除いてまれである[4].

Ⅲ 疫学

1. 動物における疫学

a. 外国での疫学

近年，西ドイツで591農場3,200頭のウシを対象にして行われた疫学調査によると，ELISA法で13.4%，補体結合反応（CF）で8.4%が陽性を示し，33.3%の農場が陽性であったとしている．とくに繁殖障害の病歴をもつウシは84.4～94.9%と，病歴のないウシが5.3～15.4%の陽性であったのに対し非常に高率で，しかも繁殖障害をもつウシの約80%が *C. burnetii* を排菌していたという[5]．畜産業において重大な生産病であり，かつ公衆衛生上の問題でもある．

b. 日本国内での疫学

わが国においては，1954年に46県のウシ983頭中11頭にCF抗体が認められたとの報告がみられる．以後はほとんど積極的な疫学調査はされていなかったが，1990年代以降に，家畜，野生動物，愛玩動物の抗体調査が行われ，高率に抗体を保有することが明らかになってきた[6)~8)]．ただし，これらの報告の抗体陽性基準が間接蛍光抗体法（IF法）で16倍と低く，抗体保有が過去の感染を意味するのか，感染状態を意味するのか，または非特異的な抗体を検出しているのか不明な点が残されていた．

筆者らは，現在のヒトへのリスクの実態解明のため，家畜や愛玩動物からの *C. burnetii* 検出を含めた疫学調査を数年前から行っている．家畜については，検体提供について協力が得られた北海道の5牧場で飼育されているウシ431頭を対象にしIF法と一部でPCRを施行した．全体としては10.5%が *C. burnetii* 抗体陽性と判定された．しかしながら，1つの牧場においては，陽性が28.4%と他の牧場に比べて高い値を示した．当該牧場を除いた4牧場における陽性の割合は4.4%であった．ウシ全血から抽出したDNA計322検体についてreal-time PCR法にて遺伝子検出を実施したが，全検体陰性であった．抗体保有率が高かった牧場のウシではDNAについては検討できなかったため，採材時点での感染の有無は不明であるが，過去に流行等があった可能性は否定できず，地域によっては一定の感染リスクは存在する可能性が示唆された．さらに家畜の調査として2009～2012年に食肉処理された全国のウシ525頭から *C. burnetii* 遺伝子は検出されず，日高地方の放牧ウマ87頭からは *C. burnetii* 遺伝子は検出されなかった．

ペットでは，血液サンプルからのDNA検出と抗体保有率で調査した．イヌでは28都道府県で採取された1,098頭の血清1,098検体と，うち25都道府県の血液からの抽出DNAサンプル986検体を用いた．また，ネコでは30都道府県で採取された582匹の血清サンプルについて抗体価測定を行った．イヌでは *C. burnetii* DNAが検出された検体はなかった．またIF法で64倍を抗体陽性基準とすると，イヌでは2.1%の抗体保有率を示し，ネコでは6.2%の抗体保有率を示した．しかし全国のネコ1,762匹の全血からは *C. burnetii* 遺伝子は検出されなかった．今回の調査ではいずれもこれまでの報告より低い抗体保有率を示したことから，イヌ，ネコにおけるQ熱の関与は示唆されるものの，以前の報告ほど高率ではなく，健康なペットからヒトへの感染リスクも現状ではあまり高くないことが示された．また宿主と目されるマダニについて岡山県で採取された802匹からは *C. burnetii* 遺伝子は検出されなかった．以上から，国内における *C. burnetii* 感染の環境リスクは高くないものと考えられた．

2. ヒトにおける疫学

a. 外国での疫学

C. burnetii は欧米においては市中肺炎の数%程度を占める一般的な起炎菌としてよく認識さ

れているが，本疾患の国内における実態や病像に関しては疫学的なデータが不足している．

世界的には多くの報告がみられる．オーストラリアでは年間に数百例，英国では11年間に1,656例，米国では28年間に1,164例，スイスでは年間30～90例，ドイツでは年間150～200例で100万人当たり0.1～3.1例，フランスでは人口10万人当たり0.58例のQ熱患者が発生しているとの報告がある．

またQ熱の集団発生は初発の食肉処理場のほか羊毛処理場，乳肉加工場などで報告されている．ヒツジに由来する集団発生は1983年にスイスで報告があり，秋に山で放牧中のヒツジを里に移動した3週後に，近隣の住民415人に発生がみられた例がある[9]．また最近では，2009年にオランダでヤギなどを感染源とするQ熱の集団発生があり，2,300人が感染し少なくとも6人が死亡している．オランダ政府は感染農場のヤギの殺処分などの対策をした．オランダの乳用ヤギは23万匹で，600カ所の牧場で飼育されているが，チーズの需要の増大に伴いヤギの数が4倍に増え，飼育密度が高くなったことで感染が広がり，感染ヤギの流産中の*C. burnetii*が風で飛ばされ，周辺の市町村で患者が多く発生したとされている．

ペットでは，ネコに由来する集団発生が多く報告されており，カナダのネコ飼育者が汚染された衣類を介して職場で16人の患者発生につながった例や，1匹のネコの流産が原因で付近の住民33人に集団発生をみた例，また飼育中のネコが原因でパーティに出席した近親者14人の発生が報告されている[10)～12)]．

b．日本での疫学

わが国では，1952年WHOの依頼を受けて畜産関連に従事する健常者を対象に調査がされ，IF法による抗体調査により，901人の食肉解体処理従事者のうち22人（2.4%）が陽性であったと報告している．内訳は，獣医師275人中62人（22.5%），食肉処理業者107人中12人（12.2%），呼吸器疾患患者184人中28人（15.2%）であった．しかし，患者や病原体は確認されず，その後しばらくQ熱は日本に存在しないと考えられていた．カナダでヒツジ胎仔を扱う研究に従事し，1988年帰国後発症した留学生の報告がわが国の最初の症例である[13]．その後の国内で感染した患者では，主にペットが感染源の可能性があるとして注目されたが，特定が困難な症例が多い．Htweらは，1978～1991年に国内で集積した626人の血清で，IF法で*C. burnetii*に対する抗体保有率を調べているが，動物と接する機会の多い獣医師の血清については，275人中22.5%が陽性であり，健常者60人での1.6%に比べて抗体陽性者が多かったと報告している[7]．

一方筆者らは，2007年に発熱等の症状のない健常者（動物病院従事者）の保存血清163検体，男性68検体，女性95検体，年齢10～70代について，スクリーニングとしてELISA法にてQ熱IgG抗体を測定し，判定保留と陽性であったものについてIF法で測定したところ，すべて陰性であった．抗体判定の基準が異なるものの，現在のリスクとしては以前よりも低いものと考えられる[14]．

成人の市中肺炎における*C. burnetii*の関与については，市中肺炎の2～6%がQ熱とする疫学調査の高橋らの報告がみられている[15]．一方筆者らのプライマリケア医における市中肺炎の調査では，168例中3例（1.8%）がIF法で血清学的に陽性であったが，明らかにWestern blottingで特異抗体と思われたものは1例（0.6%）のみであった．このようにQ熱は，日本における存在が明らかであるが，多くは見逃されている可能性が高い．一方で感染源が特定されていない症例が多いことや，まだ多くの不明な点が残されている．時に輸入例もあり，筆者らは，オーストラリアの農場視察に行った畜産関係者3人が同時に感染した急性Q熱の輸入症例や国内発症の肝炎型を経験している[16]．

表2　Q熱の確定診断（届出基準）

検査方法	検査材料
分離・同定による病原体の検出 PCR法による病原体の遺伝子の検出	血液
間接蛍光抗体法による抗体の検出（単一血清でIgM抗体64倍以上もしくはIgG抗体256倍以上，またはペア血清による抗体陽転もしくは抗体価の有意の上昇）	血清

今後も症例の蓄積と感染源の調査が望まれる．

Ⅳ 感染症法による取り扱い

Q熱は，1999年施行の感染症法で第4類全数届出感染症に指定され，2003年の改正後は新4類として規定された疾患であり，本症を診断した医師は，ただちに最寄りの保健所に届け出ることになっている．1999～2011年まで毎年それぞれ数人から40人程度が報告されている．患者（確定例）については，2003年の届出基準の見直しによって以下のように改訂されている．「医師は，臨床的特徴を有する者を診察した結果，症状や所見からQ熱が疑われ，かつ，次の表の左欄に掲げる検査方法により，Q熱患者と診断した場合には，法第12条第1項の規定による届出を直ちに行わなければならない．この場合において，検査材料は，同欄に掲げる検査方法の区分ごとに，それぞれ同表の右欄に定めるもののいずれかを用いること．」この検査法と届出基準の見直しの結果，以前は低抗体価（IgM16～32倍，IgG16～128倍）の症例が多く届けられていたものが，確定例から除外されたことによって，報告数の減少につながっているものと考えられる．

Ⅴ 病態，症状

病態は大きく急性型と慢性型に分けられる．急性型の潜伏期は一般的には2～3週間で，感染量が多いと短くなる．症状は発熱，頭痛，筋肉痛，全身倦怠感，呼吸器症状などで，インフルエンザ様である．主症状が肺炎，肝炎，あるいは発熱主体等で，「肺炎型」「肝炎型」「不明熱型」の3つのパターンがみられ，その臨床像は多彩であり，不顕性感染も多く，ほとんど特徴的なものはないといえる．症状として皮疹がみられることはまれである．検査所見では，CRP，肝酵素（AST，ALT）の上昇，血小板減少，貧血などがみられる．また，急性型の2～10%は心内膜炎を主徴とする慢性型に移行するといわれており，適切な治療をしないと難治性となる．海外では，急性Q熱患者が回復後しばらくして倦怠感，不眠，関節痛などを訴え，数カ月～数年もの間持続し，急性Q熱後症候群と診断される症例が報告されているが，まだ実態は不明な点が多い．

Ⅵ 診断

Q熱の診断法には病原体検出法と血清抗体測定法がある（表2）．

1．病原体検出法

病原体検出法には，分離培養法と抗原検出法，遺伝子検出法がある．なお，病原体検査マニュアル（国立感染症研究所・地方衛生研究所編）に現時点で推奨されるQ熱検査の詳細を記載しているので参照されたい．国立感染症研究所のHPでも閲覧可能である．

a．分離培養法

急性期のヒト材料（血液，血清，咽頭スワブ等）から分離をすることがもっとも信頼性が高いが，実験小動物や発育鶏卵，培養細胞などを用いるため，煩雑で経験を要する．動物からの

材料では血液，血清，乳，脾臓，胎児，胎盤，膣拭い液などが用いられる．発育鶏卵を用いる場合は，卵黄嚢内接種によって菌を増殖させる．細胞培養ではBGM（buffalo green monkey：アフリカミドリザル腎臓）細胞などを用いる．これらの操作はいずれもP3レベルの感染実験が可能な設備を要するため施行可能な施設は限られる．

b．抗原検出法

菌を含むと思われる臨床検体を直接Gimenez染色やギムザ染色，あるいは免疫蛍光抗体法で染色することは可能ではあるが，通常の診断としては不確実であり実際にはまず行われない．上記の分離培養後の同定としての使用にほぼ限定される．

c．遺伝子検出法

PCR法による特異遺伝子の検出がもっとも感度と特異性に優れており有用な方法である．現在はもっとも感度・特異性に優れた*com*1遺伝子が主に利用されている．筆者らは，主に全血のバフィーコート分画から検出を行っており，急性極期には血清中からの検出も可能である[17]．

2．血清抗体測定法

a．間接蛍光抗体法（IF法）

血清抗体価測定は標準法としてはIF法が推奨される．急性型では，まずⅡ相菌に対する抗体が上昇し，その後Ⅰ相菌に対する抗体が上昇する．一般にⅠ相菌よりⅡ相菌に対する抗体価が高くなるため，急性型の確定診断にはⅡ相菌を主に用い，場合によっては双方を用いて，原則的には急性期と回復期のペア血清でのIgG抗体価の4倍上昇を認めることやIgMの高値（64倍以上）やIgG高値（256倍）の証明によって行われる[17]．抗体価は最初の感染から数カ月～数年持続するため，単独血清での判定は難しいことが多い．またペア血清での有意な上昇がみられるまでにかなり長期間を要する例もあり，Q熱を強く疑う例では経過を追って抗体を測定することが望ましい[17][18]．一方，慢性型の確定診断では，Ⅰ相菌およびⅡ相菌に対する高い抗体価がみられ，一般にⅠ相菌の抗体価がⅡ相菌の抗体価より高いことから判定される．また，急性Q熱後症候群の患者では全般的に抗体価が低めであるといわれている．

Ⅶ 治療と予防

急性Q熱の多くは2週間程度で自然治癒し，死亡例はまれであるが，抗菌薬による治療を発症から3日以内に行うと有熱期間の短縮や慢性化の予防に効果ある．急性Q熱の第1選択薬はテトラサイクリン系抗菌薬（ミノサイクリン・ドキシサイクリン）で，第2選択薬としてマクロライド系が用いられるが，重症例では無効の報告もある．β-ラクタム薬はほぼ無効である．他にはリファンピシン，キノロン薬も有効とされる．テトラサイクリンやキノロン薬では耐性株の報告がみられている．菌の陰性化を期待するためには解熱後も2週間は投与を続ける必要がある．仮に再燃したら，すぐに投薬を再開することが重要である．

これまで*C. burnetii*に対する最小発育阻止濃度（MIC）の成績は乏しかったが，Rolainらは，ドキシサイクリンとニューキノロン薬（モキシフロキサシン，オフロキサシン，ペフロキサシン）の臨床分離*C. burnetii*株に対するMICを比較して，ほぼ同等の抗菌力であったとし，また彼らはテリスロマイシンの抗菌力はエリスロマイシンより優れていると報告している[19]．Gikasらも，同じくドキシサイクリンとニューキノロン薬（オフロキサシン，ペフロキサシン，トラバフロキサシン），マクロライド薬（クラリスロマイシン）の*C. burnetii*に対するMICを比較しているが，いずれも同等の抗菌力を示すと報告した．さらにGikasらは，急性Q熱症例に対して抗菌薬治療を行い，解熱するまでの期間を検討したプロスペクティブな研究成績を報告しているが，ドキシサイクリン，エリ