Outbreak of Occupational Brucellosis in a Laboratory Technician at Her Royal Highness Princess Sirindhorn Medical Center, Srinakharinwirot University, Thailand

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Objective: *To document laboratory transmission of brucellosis and identify the likely mechanism of transmission of brucellosis at Her Royal Highness (HRH) Princess Sirindhorn Medical Center, Thailand.*
Material and Method: *Using small subunit ribosomal RNA (rRNA) sequencing technique to analyze Brucella melitensis cultured from the first 2 patients of the hospital and an infected laboratory technician, and using brucellosis serologic test to rule out infections in all other involved technicians.*
Results: *We had encountered the first 2 cases of brucellosis. Both had infected from community exposure with goat. The first case had pancreatic abscess and spinal bone involvement with a positive blood culture. The second case presented with fever of unknown origin and had a positive blood culture. A few weeks later, 1 of our laboratory technicians presented with fever, myalgia and fatigue. Blood culture grew B. melitensis. He never had any associated community-acquired risk factors for brucellosis. The presumed mechanism of transmission was an inhalation while taking photographs of the bacterial plate of the first patient. B. melitensis identified from our laboratory technician and both patients were analyzed based on 16S-23S rRNA intergenic transcribed spacer (ITS) region. Results of 16S-23S rRNA ITS sequence testing confirmed a match from all patients and laboratory technician's isolate. All other 10 potentially exposed laboratory technicians were asymptomatic. A brucellosis serologic test was negative in all non-infected technicians but was only positive in the 1 infected technician.*
Conclusion: *This is the first report in Thailand of occupational brucellosis transmitted in microbiologic laboratory. The most likely mechanism is air-borne inhalation of bacterial organisms on culture media in the absence of adequate precautions. Laboratory technicians should handle Brucella cultivation with caution utilizing appropriate measures to prevent inhalation.*

Keyword: *Outbreak, Brucella melitensis, Epidemic investigation, Laboratory transmission*

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Brucella species, the causative agents of brucellosis, are pathogenic to a variety of domesticated and wild animals. Brucellosis has been widely documented for transmission in a community and a hospital especially in the setting of laboratory technicians[1-3]. Infections in humans generally result from (i) transmission via the gastrointestinal route by the consumption of unpasteurized dairy products[4] and contaminated meat[5], (ii) airborne transmission in animal husbandry by inhaling dust contaminated by aborted tissues[6], and (iii) transmission caused by laboratory-associated exposure to aerosols[1,2]. In the event of a bioterror attack, the preferred method of dissemination would most likely be via aerosol[7].

Her Royal Highness (HRH) Princess Sirindhorn Medical Center, Thailand had encountered with a possible outbreak in our medical center laboratory followed the first 2 medical center cases. Episode investigation was performed after evidence of laboratory infection.

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Material and Method

An outbreak of brucellosis was confirmed with the first 2 cases of the hospital and the infected technician identification. Using small subunit ribosomal RNA (rRNA) sequencing technique to analyze *Brucella*

melitensis cultured from the first 2 patients and the infected laboratory technician to identify the most likely transmission pathway and using brucellosis serologic test to exclude infections in all other involved technicians.

DNA preparation

DNA extractions were performed using Qiagen QIA amp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions[8]. For bacterial isolates, DNA was extracted from 1 loopful of bacteria grown for 36 hours on chocolate blood agar (CA) and eluted in 400 µL of buffer AE. DNA concentrations were measured by spectrophotometry and adjusted to a concentration of 50 ng/µL. For blood samples, DNA was extracted from 200 µL of EDTA anticoagulated whole blood and eluted in 100 µL of buffer. For tissue samples, DNA was extracted from thirty 5-µm-thick sections obtained from archived paraffin-embedded tissues and eluted in 200 µL of buffer. DNA samples were stored at -80°C until used in PCR and real-time PCR experiments.

PCR amplification and sequencing of 16S-23S rRNA spacer

The 16S-23S rRNA spacer was amplified by polymerase chain reaction (PCR) with the primers F2 and R1 (Table 1)[8], were constructed based on the result of multiple alignments of 16S and 23S rRNA sequences from *B. abortus* (Gen Bank accession numbers AE017224 and NC006933) and the most similar sequences strains (*B. melitensis*, *B. suis*, *B. ovis* and *B. canis*) using CLUSTAL W version 2.0.2. The PCR mixture was consisted of 1X PCR buffer, 1.5 mM MgCl2, 10 mM dNTPS, 20 µM of primer and 3 unit of *Taq* DNA polymerase. The sterile distilled water was used to make the total volume of 25 µL, the reaction was performed by using the Peltier Thermal cycle (MJ Research, PTC-200). The PCR condition was preheated at 95°C for 3 min. PCR amplification was performed for 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. The PCR fragment was analyzed by electrophoresis on 1% agarose gel. The PCR product was eluted and purified by using QIAGEN PCR purification kit (QIAGEN) and then cloning into plasmid vector. The sequences of insert PCR product was determined by using Big Dye Terminator Cycle sequencing procedure and analyzed using ABI PRISM 377 (Perkin Elmer). The nucleotide sequencing data were detected by input the data to BLAST program from http://www.ncbi.nlm.nih.gov/BLAST. The nucleotide sequences of 16S-23S rRNA spacer of *Brucella* species diagnostic was compared to those of other *Brucella* species that have been previously submitted in Gen Bank.

Real-time PCR and High resolution melt (HRM)

The primers list in Table 1 were designed to 16S-23S ITS region of *Brucella*. This region was confirmed by sequencing using constructed plasmid from conventional PCR amplification method. The real-time PCR reaction was carried out in a 25 µL total volume containing: 10 µL of 2X QuntiMix Probe (1X PCR buffer, 1.5 mM $MgCl_2$, 10 mM of each of the four dNTPs, 1 unit of *Taq* DNA polymerase) (Biotools, Germany), 2 µM of SYTO 9 (1: 100) and 20 µM primers. The intercalating dye used was SYTO 9 (Invitrogen, USA). The real-time PCR reactions and HRM analysis were performed on Rotor-Gene 6000™ (Corbett Research, Cybeles, Thailand). The real-time PCR profile comprised of one initial cycle of 95°C for 2 minutes then follow by 40 cycles of 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. After real-time PCR amplification was completed, HRM was performed using melting profile from 72 to 95°C rising at 0.2°C per second. The melting curves were normalized by software provided with the Rotor-GeneTM 6000 according to previous report.

Serologic test for Brucella

The antibodies to *B. melitensis* were done by using agglutination tests (New Market Laboratories Ltd, Kentford, Suffolk, CB87PN, United Kingdom)[9]. The test was done by using a pipette, dispensing 0.08 mL, 0.04 mL, 0.02 mL, 0.01 mL and 0.005 mL of undiluted serum to mix with undiluted *B. melitensis*

Table 1. The nucleotide sequences and locations of primers[8]

Primer	Sequence (5' to 3')	Location
F2 (Forward)	CTAACCGCAAGGAGGCAGGC	*B. abortus* 16S rRNA
R1 (Reverse)	CGCCAGCCGCATAGCAGGGT	*B. abortus* 23S rRNA

antigen suspension. After one minute, agglutination reaction was observed. The positive titer in 0.08 mL, 0.04 mL, 0.02 mL, 0.01 mL and 0.005 mL solutions refer to positive titers of 1: 20, 1: 40, 1: 80, 1: 160 and 1: 320, respectively.

Results

The 2 patients and the infected technician were identified and reviewed which confirmed for an outbreak of brucellosis in our hospital. The first case was a 66 year old female who owned a goat farm. She presented with fever, back and abdominal pain for a few weeks. Blood cultures were positive on the fourth-day of incubation, showed Gram-negative cocco-bacilli. The positive blood cultures had also been drawn for subculturing in blood agar (BA), CA, and Mac Conkey (MAC) agar. After overnight incubation, the pin-point colonies have been observed on both BA and CA but not on MAC agar (Fig. 1). The heavy inoculation of colonies had also been applied in Christensen, urea slant test. The rapid reaction of has been observed within 1 minute[10,11]. They were further identified as *B. melitensis* (Fig. 2). Magnetic resonance imaging (MRI) scan of the thoracic spine showed early destruction of T9/T10 vertebral body and intervertebral disc with adjacent end plate. The findings were consistent with discitis and spondylitis (Fig. 3a). Computerized tomography (CT) scan of abdomen suggested pancreatic abscess (Fig. 3b). She had been received the treatment with doxycycline (100 mg every 12 hours) and streptomycin (750 mg once daily) for a few weeks and followed with doxycycline (100 mg every 12 hours) and rifampicin (600 mg daily) for 3 months with complete improvement.

The second case was a 71 year old female who was hospitalized a few weeks following the first case. She presented with fever of unknown origin for a few weeks. A few days after incubation, her blood culture grew *B. melitensis*. She lived near a goat farm and had taken the goat milk. She had been received the treatment with doxycycline (100 mg every 12 hours) and rifampicin (600 mg daily) for 6 weeks with complete improvement.

A few weeks later, 1 of our laboratory technicians presented with fever, myalgia and fatigue. His blood culture grew *B. melitensis*. He never had any associated community-acquired risk factors for brucellosis. He received treatment with doxycycline (100 mg every 12 hours) and rifampicin (600 mg daily) for 6 weeks with improvement.

After the diagnosis of the laboratory technician, HRH Princess Sirindhorn Medical Center infection control team has investigated the episode. The plan for investigation is presented in Diagram 1.

We then had the through interview with the infected technician for possible mechanisms of transmission. The through interview with infected technician reviewed the 2 possibilities of transmission. The mechanism of transmission possibly occurred

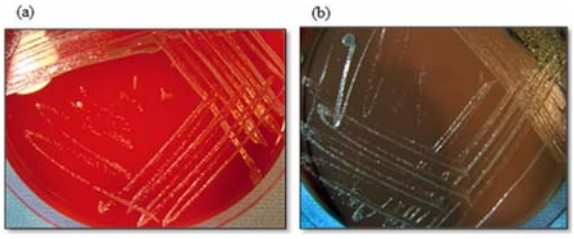

Fig. 1 Colonies morphology on (a) blood agar and (b) Chocolate agar respectively.

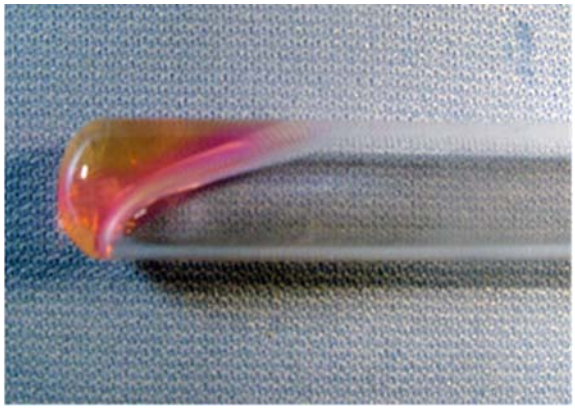

Fig. 2 Christensen' urea slant test shows positive result within 1 minute.

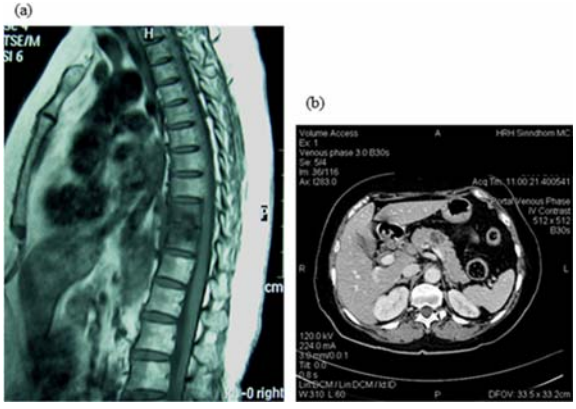

Fig. 3 A) MRI scan of thoracic spine. B) CT scan of the abdomen.

upon taking photography of the bacterial plate of the first patient (outside microbiologic hood without airborne precaution) or during early identification of bacterial plate of the second patient (total of 11 technicians exposure to the bacterial plate during that early identification).

In order to confirm the mechanism of transmission, *B. melitensis* identified from our laboratory technician and both patients were analyzed based on the 16S-23S rRNA ITS region. The percent homology among nucleotide sequences of the 16S-23S rRNA ITS sequences from three *B. melitensis* isolates were 99% homology, suggested a match from the first patient and laboratory technician's isolates. Upon PCR analysis of the 16S-23S rRNA ITS sequences[12,13], the 1,200 bp fragments were obtained from all 3 *Brucella* DNA specimens (Fig. 4). The similarity of the nucleic acid content(s) of those 1,200 bp PCR products showed high degree of genetic homology.

The specificity of primers used in the experiment was confirmed by the real-time PCR and high resolution melt (HRM) analysis. Identification of all *Brucella* genomic DNAs based on real-time PCR amplification followed by HRM analysis of the 16S-23S rRNA ITS sequences indicated that they were closely related species (Fig. 5). The sequence analysis of the insert revealed very high homology (>99%) of *B. melitensis*, *B. abortus* and *B. suis*.

There were other 10 technicians involved in early identification of bacterial plate of the second patient. They were all asymptomatic. Serologic test for *Brucella* was tested to rule out the possible infection. The sera from both patients and all technicians were tested for *Brucella* antibody. The result showed 1: 80 (positive) only in both patients and the infected technician. All other results were negative (<1: 20).

Discussion

The genus *Brucella* is comprised of Gram-negative, facultative, intracellular pathogens[14]. The phenotypic characteristics, antigenic variation, and prevalence of infection in different animal hosts have resulted in the initial recognition of 6 species including *B. abortus* (cattle), *B. melitensis* (goats/sheep), *B. suis* (swine), *B. canis* (dogs), *B. ovis* (rams), and *B. neotomae* (desert rats)[15,16]. Recently 2 *Brucella* strains from different marine mammals have been reported[17,18] and the names *B. pinnipediae* (seal/otter) and *B. cetaceae* (porpoise/whale) have been proposed[19]. DNA hybridization analyses indicate a high level of homology among the *Brucellae*, suggesting that the genus *Brucella* may comprise only one species with several biovars[20].

Brucellosis impacts public health and agricultural economies worldwide because of its high infectivity rate[15]. *Brucella* spp have also long been considered a potential biological weapon[7], and currently, with the renewed threat of biological warfare

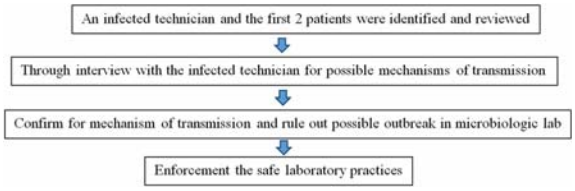

Diagram 1. Plan for epidemic investigation.

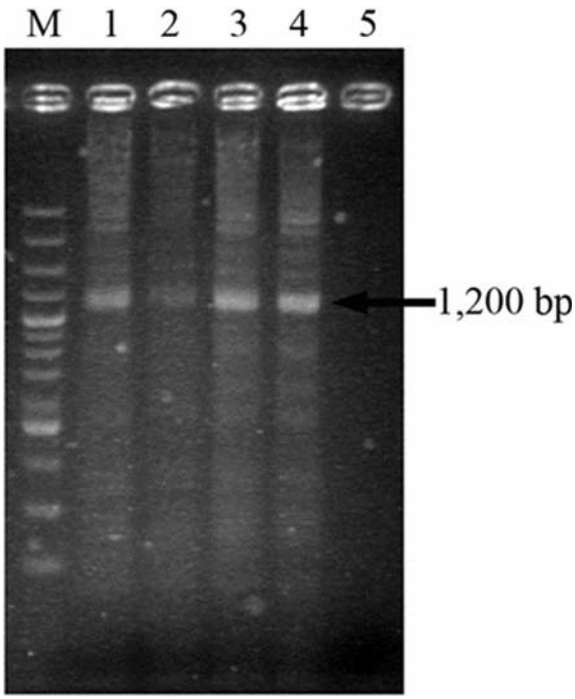

Fig. 4 Detection of 16S-23S rRNA intergenic transcribed spacer (ITS) of Brucella DNA from patients with brucellosis by using PCR. A target sequence of 1,200 bp was amplified in Brucella DNA. The amplified fragments were separated by electrophoresis on a 1% agarose gel, stained with etidium bromide and photographed under UV light. Lane M, DNA marker, 100 bp + plus (Fermentas®); Lane 1, *Brucella* spp. DNA from patient 1; Lane 2 and 3, *Brucella* spp. DNA from patient 2; Lane 4, *Brucella* spp. DNA from patient 3; Lane 5, distill water.

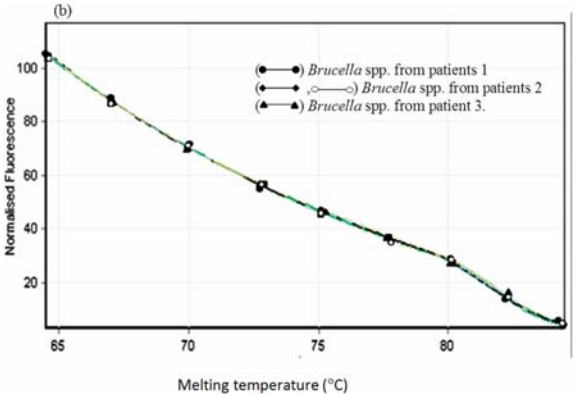

Fig. 5 Detection amplified curves of 16S-23S rRNA ITS-derived primers using SYTO 9 (1:100) with Rotor-Gene 6000™ (Corbett Research, Cybeles, Thailand) followed byHRM analysis using Rotor-Gene 6000 series 1.7 software.

and agricultural terrorism, *B. melitensis*, *B. suis*, and *B. abortus* are listed as category B bio-threat agents by the Centers for Disease Control and Prevention Strategic Planning Group[21,22].

Because of the limited availability of animal vaccines, cost of animal inoculations, a lack of vaccines for human use[15], and its low infectious dose for humans, human brucellosis is endemic in many parts of the world, including the Mediterranean region, Latin America, Asia, and Africa. The reported incidence varies from <0.01 to >200 per 100,000 populations. Human brucellosis is rare in the United States, with approximately 100 human cases reported per year, mostly caused by the consumption of unpasteurized dairy products and, to a lesser degree, occupational exposure[23].

The genus *Brucella* consists of a highly conserved group of organisms. The identification of the four *Brucella* species pathogenic in humans (*B. melitensis*, *B. abortus*, *B. suis*, and *B. canis*) is problematic for many clinical laboratories that depend primarily on serology and phenotypic characteristics to differentiate species. PCR amplification of the 16S-23S rRNA ITS region was evaluated for species-specific polymorphism. The spacer region often varies not only in sequence but also in length among species. Thus, simple visual observation of the sizes of PCR products is sufficient for species differentiation. Rijpens et al reported that the 16S-23S rRNA spacer of *Brucella* comprises about 800 bp after PCR amplification[24]. Sequence analysis of the insert revealed very high homology (>99%) of *B. melitensis*, *B. abortus*, *B. suis*.

In this study, amplification of the 16S-23S rRNA spacer region generated bands estimated 1,200 bp (Fig. 4). The similarity of the PCR products for the *Brucella* DNA samples provides further evidence for their high degree of genetic relatedness. However, these PCR products were unique to *Brucella*, allowing them to be readily distinguished from other Gram-negative bacteria. Further investigation, these PCR product fragments overlap of the 16S and 23S rRNA region were eluted, purified, cloning into plasmid vector and then sequences of PCR product. The nucleotide sequences of 16S-23S rRNA spacer of *Brucella* spp in this study was compared to those of other *Brucella* spp (including, *B. melitensis*, *B. abortus*, *B. suis* and *B. canis*) that have been previously submitted in GenBank.

In addition, 16S-23S rRNA ITS sequences of 3 *Brucella* of clinical isolates were determined by using phylogenetic analysis. In the same time, four genomic DNAs samples of *Brucella* spp were determined using real-time PCR amplification followed by HRM analysis, all *Brucella* DNA generated overlap amplicon melting curves HRM. Therefore, *Brucella* DNA from 3 patients exhibited identical of 16S-23S rRNA ITS sequences. In conclusion, PCR profiles may now be used as a confirmatory test for isolates that have been presumptively identified by conventional physiological tests. It is possible that, with more extensive evaluation in the clinical laboratory, PCR profiling might become a primary method for the designation of isolates as *Brucella*. However, the real time PCR assay is suitable for diagnosis and phylogenetic relationships assay of *Brucella* species.

Although the genes from spacer region 16S-23S could not confirm the origination of the technician bacteria since all 3 bacteria seemed to be the same, all the other evidence should support that the transmission of brucellosis was from the first patient to the laboratory technician via inhalation of the bacterial colonies after exposure of the plate outside the microbiologic hood while taking the photography of the plate. If the technician got brucellosis from the second patient, some other involved technicians should also have it. The serologic test helped in this interpretation since it was positive in only two patients and the infected technician.

According to previous laboratory transmissions reported, the mechanisms were sniffing of culture plates, spilling blood culture bottles, mucosa or cutaneous contacting by organism-containing suspensions, aerosol exposing from broken centrifuge

tubes, or routine laboratory working with Brucella cultures outside of microbiological hood[1,2,25-28]. The infection control team had concluded the outbreak investigation and went back to reinforce all the laboratory technicians for the safety laboratory practices which included safety handling of bacterial cultivation, avoid taking culture plate outside the microbiologic hood and using adequate protective equipment. There was no other outbreak till now.

Another interesting issue was the epidemiology of brucellosis in the community where those patients were from. Ekpanyaskul C et al had shown the serology tests of 86 persons from 3 villages in Chumpon sub-district, Ongkharak district, Nakhon Nayok province, Thailand where the first patient was from[29]. There were 45.35% of all participants who had seropositive antibodies to *B. melitensis*[29]. Multivariate analysis indicated that factors associated with seropositive titers were highly related to contact with labored or aborted goats and the consumption of raw goat products[29]. So, health education in this specific issue was needed in the community where goat farm was involved.

Conclusion

This is the first report in Thailand of occupational brucellosis transmitted in microbiologic laboratory. The most likely mechanism is air-borne inhalation of bacterial organisms on culture media in the absence of adequate precautions. Laboratory technicians should handle *Brucella* cultivation with caution utilizing appropriate measures to prevent inhalation. It might be important that *Brucella* cultivation should only be reserved in the microbiologic hood.

What is already known on this topic?

Brucellosis can be transmitted in microbiologic laboratory.

What this study adds?

This is the first report in Thailand of occupational brucellosis transmitted in microbiologic laboratory. The most likely transmission mechanism was an air-borne inhalation of bacterial organisms on culture media in the absence of adequate precautions. Laboratory technicians should handle *Brucella* cultivation with caution utilizing appropriate measures to prevent inhalation. Epidemic investigation for each institution was important to set up specific recommendation for its situation.

Potential conflicts of interest

None.

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การระบาดของโรคบลูเซลโลซิสจากการปฏิบัติงานของเจ้าหน้าที่ห้องปฏิบัติการที่โรงพยาบาลศูนย์การแพทย์สมเด็จพระเทพรัตนราชสุดาฯ สยามบรมราชกุมารี มหาวิทยาลัยศรีนครินทรวิโรฒ ประเทศไทย

วรพจน์ ตันติศิริวัฒน์, วันชัย บุพพันเหรัญ, สมชาย สันติวัฒนกุล, โกสุม จันทร์ศิริ

วัตถุประสงค์: เพื่อยืนยันการระบาดของโรคบลูเซลโลซิสทางห้องปฏิบัติการและค้นหากระบวนการของการระบาดของโรคบลูเซลโลซิส ที่โรงพยาบาลศูนย์การแพทย์สมเด็จพระเทพรัตนราชสุดาฯ สยามบรมราชกุมารี ประเทศไทย

วัสดุและวิธีการ: ใช้เทคนิค small subunit ribosomal RNA sequencing เพื่อวิเคราะห์ Brucella melitensis ที่เพาะเชื้อได้จากผู้ป่วย 2 คนแรกของโรงพยาบาลและที่ได้จากเจ้าหน้าที่ห้องปฏิบัติการและใช้ brucellosis serologic test เพื่อตัดประเด็นของการติดเชื้อของเจ้าหน้าที่ห้องปฏิบัติการที่เกี่ยวข้องทั้งหมด

ผลการศึกษา: เราพบการติดเชื้อบลูเซลโลซิส 2 คนแรก ซึ่งติดต่อจากชุมชนโดยมีการติดต่อโดยสัมผัสกับแพะ ผู้ป่วยรายแรกมีฝีที่ตับอ่อนและมีการติดเชื้อที่กระดูกสันหลังร่วมกับการติดเชื้อในกระแสโลหิต ผู้ป่วยรายที่สองเป็นไข้โดยไม่ทราบสาเหตุและมีการติดเชื้อในกระแสโลหิต หลังจากนั้นไม่กี่สัปดาห์เจ้าหน้าที่ห้องปฏิบัติการคนหนึ่งมีอาการไข้ ปวดเมื่อยตามตัวและอ่อนเพลีย ผลการเพาะเชื้อในเลือดพบ B. melitensis เจ้าหน้าที่โรงพยาบาลดังกล่าวไม่เคยมีความเสี่ยงใด ๆที่จะติดเชื้อบลูเซลโลซิสจากชุมชน กระบวนการการติดเชื้อคาดว่าน่าจะเป็นจากการสูดดมเชื้อเข้าไประหว่างที่ทำการถ่ายรูปจานเพาะเชื้อของผู้ป่วยรายแรก B. melitensis ที่เพาะได้จากในเจ้าหน้าที่ห้องปฏิบัติการและผู้ป่วยทั้งสองรายถูกนำไปวิเคราะห์โดย 16S-23S rRNA intergenic transcribed spacer (ITS) region ผลการวิเคราะห์โดย 16S-23S rRNA ITS ยืนยันว่าเชื้อของเจ้าหน้าที่ห้องปฏิบัติการเข้ากันได้กับเชื้อจากผู้ป่วยทั้งสองราย เจ้าหน้าที่ห้องปฏิบัติการที่เหลืออีก 10 คน ที่มีโอกาสสัมผัสเชื้อไม่มีอาการอะไร ผล brucellosis serologic test ของเจ้าหน้าที่ทุกคนที่ไม่ติดเชื้อมีผลเป็นลบ ในขณะที่ผลเป็นบวกในเจ้าหน้าที่ที่ติดเชื้อ

สรุป: รายงานนี้เป็นรายงานแรกในประเทศไทยที่กล่าวถึงการติดเชื้อบลูเซลโลซิสจากการปฏิบัติงานในห้องปฏิบัติการ กระบวนการการติดต่อน่าจะเกิดจากการติดเชื้อทางอากาศโดยการสูดดมเชื้อจากจานเพาะเชื้อโดยขาดการป้องกันอย่างเหมาะสม เจ้าหน้าที่ห้องปฏิบัติการจะต้องระมัดระวังตัวอย่างยิ่งในการเพาะเชื้อบลูเซลโลซิสและต้องมีวิธีป้องกันการสูดดมเชื้อเข้าไปอย่างเหมาะสม