Original Article

Effectiveness of sentinel rodents for surveillance of exposure to undocumented bacterial pathogens in animal research facility

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Abstract

The use of sentinel animals in animal research facilities is the cornerstone of animal health monitoring for exposure to natural pathogens of laboratory animals. This is because infection with these pathogens produces no overt signs of disease yet the infection may affect the outcome of research utilising these animals. One of the important pathogens includes *Klebsiella pneumoniae*, whose prevalence can be high in laboratory animals. This study sought to document the prevalence of *K. pneumoniae* and other undocumented bacterial pathogens in laboratory rodents for a 3-year period. *K. pneumoniae* and *Chryseobacterium gleum* were isolated from sentinel ICR mice housed in three different satellite animal laboratories, suggesting the effectiveness of the sentinel program. A novel strain of *K. pneumoniae* ST3125 was recovered from the gastrointestinal tract of the ICR mouse. β-lactamase and virulence genes were detected among *K. pneumoniae* and *C. gleum* strains, suggesting the acquisition of these genes from the users of the animal research facilities. Examination of the animal housing environment, feed and water specimens however, returned negative for the presence of *K. pneumoniae* and *C. gleum* suggesting that current hygiene practices were adequate in controlling transmission from the environment. Nevertheless, stringent hygiene practices and infection control protocols have to be applied in animal facilities to prevent the colonization and spread of pathogens capable of distorting experimental results.

Keywords: *Chryseobacterium gleum*, infectious disease, *Klebsiella pneumoniae*, laboratory animals, Malaysia

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Introduction

The increasing demand for laboratory animals, particularly rodents in biomedical research and biotechnology, reflects the rapid development of medicine for human and veterinary applications. The welfare of laboratory rodents has to be safeguarded to minimise discomfort, since stress may lead to non-specific physiological effects which can jeopardise experimental results (Baumans, 2005). Additionally, researchers in Malaysia have moral and binding legal obligations under the Biosafety Act 2007 and the Animal Welfare Act 2015 to ensure that experimentation done using these animals is performed as humanely as possible and in compliance with all ethical standards (Biosafety Act 2007; Animal Welfare Bill 2015). The animal research facility sentinel program was introduced to actively monitor the microbiological status of laboratory rodent colonies so as to ensure that the animals in the facility are not exposed to undocumented pathogens (Brielmeier et al., 2006).

Guidelines for the design of sentinel programs were published by the Federation of European Laboratory Animal Science Associations (FELASA) Working Group on Health Monitoring of Rodent and Rabbit Colonies in June 2001 (Nicklas et al., 2002). It is of vital importance to use quality animals with known biological characteristics as research using animals so as to ensure the reliability and the reproducibility of experimental results. Exposure to infectious diseases will adversely affect the physiology of the animals, inducing changes in behavior, growth rate, relative organ weight and immune response (Nicklas et al., 2002). The negative influence of the mouse parvovirus on mice' reproductive and immune functions (Carty, 2008), is a case in point. Exposing sentinel animals to soiled bedding is a common method for detecting viral and bacterial infections in rodents (Manuel et al., 2008). Amongst the viruses and bacteria of interest which have been identified using this method are the mouse parvovirus (Watson, 2013), the mouse hepatitis virus (Smith et al., 2007), the murine norovirus (Manuel et al., 2008) and the *Helicobacter* sp. (Whary et al., 2000).

Besides the environment, laboratory animal infections may also be introduced by humans, particularly the researchers who utilize the animal facilities. Even after applying the proper personal protective equipments, the risk of commensal bacteria transmission to laboratory animals still exists (Loong et al., 2016[a]). *Klebsiella pneumoniae* is a commensal microorganism in mammals, which can also cause opportunistic infection at ectopic sites or turn pathogenic upon the acquisition of virulence genes that aid spread and cause morbidity and mortality (Pendleton et al., 2013). Thus, it is imperative to control the spread of these pathogens in the animal research facility. While it was not a requirement, we included testing for the presence of virulence genes in commensal *Escherichia coli* into our existing animal health monitoring program (Loong et al., 2016[a]). We intend to expand the examination, covering *K. pneumoniae* and other undocumented bacterial pathogens to ascertain their genetic background for the surveillance of potential infection from exogenous sources. In this study, the bacteriological status of rodents housed in the three satellite animal laboratories between the period of January 2014 and June 2017 was determined. This study aimed to detect *K. pneumoniae* and undocumented bacterial pathogens and subsequently, examine these strains to determine their genetic and phenotypic characteristics.

Materials and Methods

Animal Housing Condition: Outbred females of ICR mice and Sprague Dawley (SD) rats with health certificates, aged four to six weeks old were introduced as sentinels in three different satellite animal laboratories in the Faculty of Medicine, University of Malaya and were kept for eight weeks throughout the entire sentinel program. The rodents were housed in open or individually ventilated cages (IVC), at a room temperature of 19-21 °C, a relative humidity of 55-65 % and a 12/12 h light/dark cycle. Mice were serologically tested to be free from these pathogens; epizootic diarrhea of infant mice, mouse hepatitis virus, *Mycoplasma pulmonis*, mouse parvovirus, minute virus of mice, pneumonia virus of mice, respiratory enteric orphan virus, Sendai virus and Theiler's murine encephalomyelitis virus, while rats were serologically tested free from Kilham rat virus, *Mycoplasma pulmonis*, pneumonia virus of mice, rat coronavirus, respiratory enteric orphan virus, rat parvovirus, Sendai virus and Theiler's murine encephalomyelitis virus. The sentinel animals were housed in pairs per cage and each cage was clearly identified as sentinels. The sentinel animals were exposed to soiled bedding from each animal cage during the cage changing process. About 25 grams of soiled bedding were transferred to the sentinel animal cage. The introduction of soiled bedding to different sentinels was done each week to ensure exposure to as many animals as possible in the room, and the handling of these sentinel animals was performed after care has been provided for all other animals in the room. Overall, thirty one rats (n=31) and thirty mice (n=30) were included as sentinel animals in this 3-year study and at least two to three sentinel cages were placed in each satellite animal laboratory.

Tissue Sample Collection: After eight weeks of exposure to soiled bedding, the sentinel animals were sacrificed for full necropsy. The necropsy examination was done in a biosafety cabinet or on a clean and sanitized bench top. The rodents were anesthetized intraperitoneally prior to blood collection by terminal bleeding (Loong et al., 2016[a,b]). Rats were anesthetized with a mixture of 50 mg/kg ketamine and 5 mg/kg xylazine, while mice were anaesthetized with a mixture of 80 mg/kg ketamine and 10 mg/kg xylazine. Following this, rats were given an overdose of anesthesia or injection of euthanasia solution (pentobarbitone), whilst cervical dislocation was performed on mice to confirm death. Aseptic sampling of selected organs such as the trachea and lung, liver and the gastrointestinal tract was then performed for bacterial culture of *K. pneumoniae* and other undocumented bacterial strains.

Bacterial Culture: Protocols for bacterial culture have been described by Loong et al. (2016[a]). Pure cultures of the bacterial isolates grown on MacConkey agar were obtained and identified using 16S rDNA sequencing (Loong et al., 2016[b]). The isolated bacteria strains from this study were tested with the extended-spectrum β-lactamase (ESBL) phenotypic confirmatory method using antibiotic discs containing cefotaxime and ceftazidime with clavulanic acid on Mueller-Hinton agar (CLSI, 2012). This study and all animal handling procedures were approved by the Institutional Animal Care and Use Committee, University of Malaya (Reference no. 2013-11-12/AEU/B/WPF).

Molecular Assays: *K. pneumoniae* strains isolated from the animals were subjected to string test (Fang et al., 2004). Amplification of virulence genes; *aerobactin*, *allS*, *kfu*, *magA* and *rmpA*, was performed following protocols established by Yu et al. (2008). β-lactamase genes (*bla*$_{ACC}$, *bla*$_{ACT}$, *bla*$_{CTX-M}$, *bla*$_{DHA}$, *bla*$_{FOX}$, *bla*$_{GES}$, *bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{LAT}$, *bla*$_{MOX}$, *bla*$_{NDM}$, *bla*$_{OXA-1}$, *bla*$_{OXA-48}$, *bla*$_{PER}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{VEB}$ and *bla*$_{VIM}$) were amplified following published protocols (Loong et al., 2016[a]) and multi locus sequence typing (MLST) was performed using modified primers from Guo et al. (2015). All amplified DNA fragments were purified and sequenced in both directions, and MLST data was deposited at http://bigsdb.pasteur.fr/klebsiella/klebsiella.html. Minimum spanning trees employing the MLST data were constructed using PHYLOViZ Online (Ribeiro-Gonçalves et al., 2016) to visualise the evolutionary relationships of the *K. pneumoniae* strains isolated in this study.

Minimum Inhibitory Concentration and Transmission Electron Microscopy: The minimum inhibitory concentrations (MIC) against amikacin, cefotaxime, ceftriaxone, ceftazidime, ciprofloxacin, levofloxacin, gentamicin, imipenem, meropenem and tetracycline were determined for other undocumented bacterial strains, according to the breakpoints for non-*Enterobacteriaceae* (CLSI, 2012) using M.I.C.Evaluator Strips (Thermo Fisher Scientific, Cheshire, UK). Transmission electron microscopy was performed to observe the structural features of other undocumemted bacterial strains (Loong et al., 2017[b]). Additionally, swabs were taken from tabletops, taps, trolleys, wash basins, animal feed and drinking water (Loong et al., 2016[a]), to be cultured for *K. pneumoniae* and the undocumented bacterial strains.

Results

A total of three (n=3) *K. pneumoniae* and one (n=1) *Chryseobacterium gleum* confirmed via 16S rDNA sequencing were isolated from ICR mice in the course of the sentinel surveillance (Table 1). None of these bacterial species was detected among the laboratory rodents prior to the commencement of the sentinel program, suggesting acquisition from the satellite animal laboratories. The *K. pneumoniae* strains were isolated from the mice' gastrointestinal tracts while the uncommonly found, yellow-pigmented *C. gleum* was isolated from the liver (Table 1). *K. pneumoniae* UM-AEU320 was cultured from an ICR mouse kept in open cage, whereas the other bacterial strains were cultured from mice kept in individually ventilated cages (IVC) (Table 1).

K. pneumoniae strains UM-AEU250 and UM-AEU320 had the identical sequence type 1440 (ST1440), carried one β-lactamase gene (*bla*$_{SHV}$) and one *kfu* virulence gene (Table 2). The *K. pneumoniae* strain UM-AEU437 was characterized as a novel ST3125 carrying two β-lactamase genes (*bla*$_{SHV}$ and *bla*$_{VIM}$) and one *kfu* virulence gene (Table 2). Sequencing of the amplified β-lactamase genes revealed sequences similar to *bla*$_{SHV}$ (accession no. FJ668811) and *bla*$_{VIM}$ (accession no. HQ858608). They were all non-ESBL producers and were negative for string test (Table 2). The constructed minimum spanning trees based on *K. pneumoniae* MLST profiles suggest that ST1440 and ST3125 (novel ST) emerged respectively from ST17 (Figure 1) and ST347 (Figure 2) as they were double locus variants of their respective founders. *C. gleum* strain UM-AEU276 was tested as a non-ESBL producer although the Ambler class A ESBL, *bla*$_{CGA-1}$ (Bellais et al., 2002) was amplified from its genomic DNA (accession no. LT009413). MIC assays revealed that the *C. gleum* strain UM-AEU276 was resistant to tetracycline but susceptible to amikacin, cefotaxime, ceftriaxone, ceftazidime, ciprofloxacin, levofloxacin, gentamicin, imipenem and meropenem. Imaging of the yellow-pigmented *C. gleum* strain UM-AEU276 using transmission electron microscopy (Loong et al., 2017[b]) at 40,000x magnification displayed a rod-shaped morphology (Figure 3) similar to another member of the genus *Chryseobacterium*, *C. joostei* (Hugo et al., 2003). Cultures from the swabs taken from the animal housing environment, animal feed and drinking water were free from *K. pneumoniae* and *C. gleum*, suggesting that these organims could have originated from other sources.

Table 1 Bacteria isolated from sentinel rodents.

Strain	Mouse strain	Collection date	Organ[a]	Housing condition[b]	Satellite laboratory	Bacteria identity
UM-AEU250	ICR	February 2015	GIT	IVC	A	*K. pneumoniae*
UM-AEU276	ICR	July 2015	Liver	IVC	A	*C. gleum*
UM-AEU320	ICR	September 2015	GIT	Open cage	B	*K. pneumoniae*
UM-AEU437	ICR	June 2017	GIT	IVC	C	*K. pneumoniae*

[a]GIT = Gastrointestinal tract; [b]IVC = Individually ventilated cage

Table 2 Genetic and phenotypic features of *K. pneumoniae* isolated from sentinel rodents.

Strain	ST	ESBL-phenotype[a]	β-lactamase gene	Virulence gene
UM-AEU250	ST1440	-	bla_{SHV}	*kfu*
UM-AEU320	ST1440	-	bla_{SHV}	*kfu*
UM-AEU437	ST3125	-	bla_{SHV}, bla_{VIM}	*kfu*

[a]- = Non-ESBL producer

Discussion

The rodent health monitoring sentinel program described in this study which had been effective in detecting rare bacterial infections, as shown by the detection of the recently characterized *Bordetella pseudohinzii* (Loong et al., 2017[a]), was expanded to the surveillance of *K. pneumoniae* and other undocumented bacterial infections. Two different *K. pneumoniae* STs (ST1440 and ST3125), one of which is a novel ST3125 and *C. gleum*, were recovered in three satellite animal laboratories. The amplification of β-lactamase and virulence genes in the *K. pneumoniae* and *C. gleum* strains suggest that these strains were not part of the mice' normal flora nor from within the laboratories, but were most likely introduced externally. Commensal bacteria which form the normal flora of the mouse do not usually carry antimicrobial resistance nor virulence genes (Loong et al., 2016[a]), as carrying these genes would have a negative effect on the biological fitness of the commensals (Löhr et al., 2015).

K. pneumoniae bla_{SHV} and bla_{VIM} carrying strains are prevalent in the hospital environment worldwide (Nordmann et al., 2011; Yan et al., 2015; Kanamori et al., 2017), and those carrying the *kfu* virulence gene are associated with human liver abscesses (Zhang et al., 2015). The isolation of *C. gleum* strains from clinical patient specimens (Lo and Chang, 2014) have also increased recently, implying that the strains from this study could have originated from the hospital environment. However, the clinical *C. gleum* strain reported by Garg et al. (2015) was susceptible to tetracycline, in contrast to our strain, suggesting that *C. gleum* UM-AEU276 could be under selective tetracycline pressure. Both *K. pneumoniae* and *C. gleum* can survive on inanimate surfaces and have been found to persist in the hospital environment (Garg et al., 2015; Jin et al., 2015). Considering that *K. pneumoniae* and *C. gleum* could not be detected from the examination of the animal housing environment, feed and water, this could imply that the hygiene practices in the satellite animal laboratories were adequate for controlling transmission of infectious bacteria from the environment. Accordingly, this would also explain the relatively rare instances where undocumented bacterial strains were isolated. In addition, the bacteria culture protocols in this study would favor pathogenic strains (Loong et al., 2016[b]) over commensal bacteria which would not likely be cultured (Stewart, 2012).

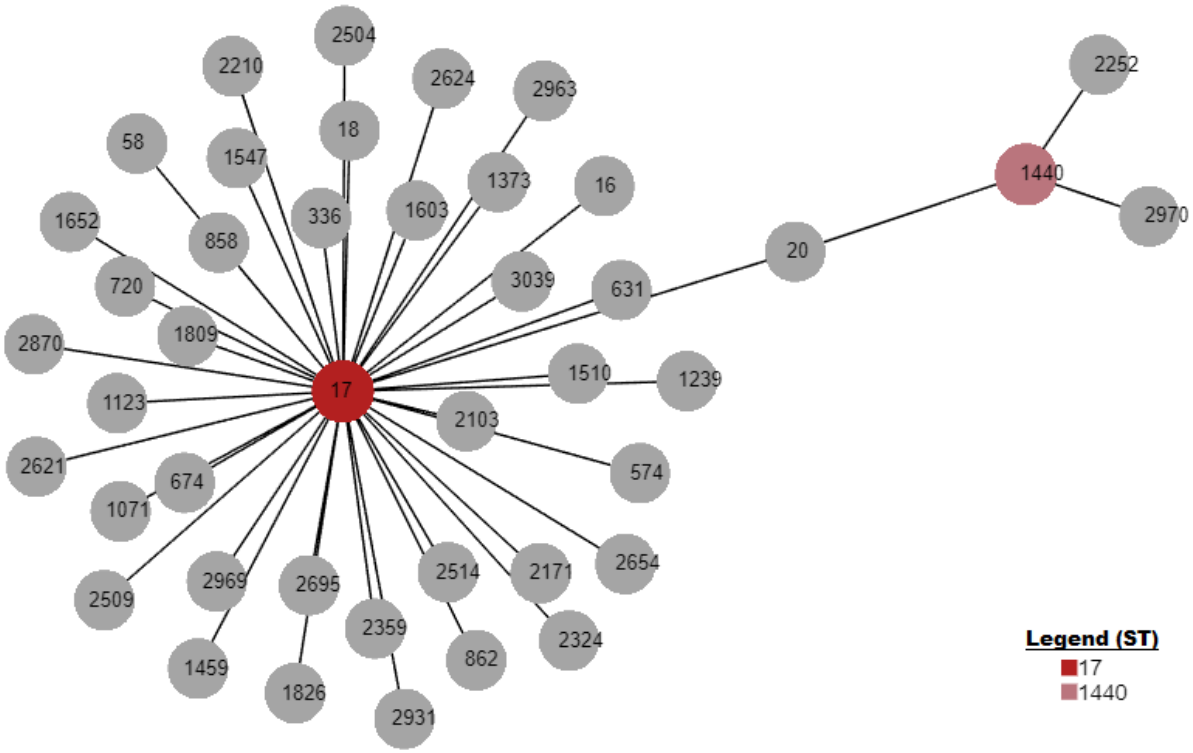

Figure 1 Minimum spanning tree showing *K. pneumoniae* ST1440 arising from the ST17-lineage. Each node represents a distinct ST and each branch represents a single locus variant. ST1440 is a double locus variant of ST17. Branch lengths are not proportional to genetic distance.

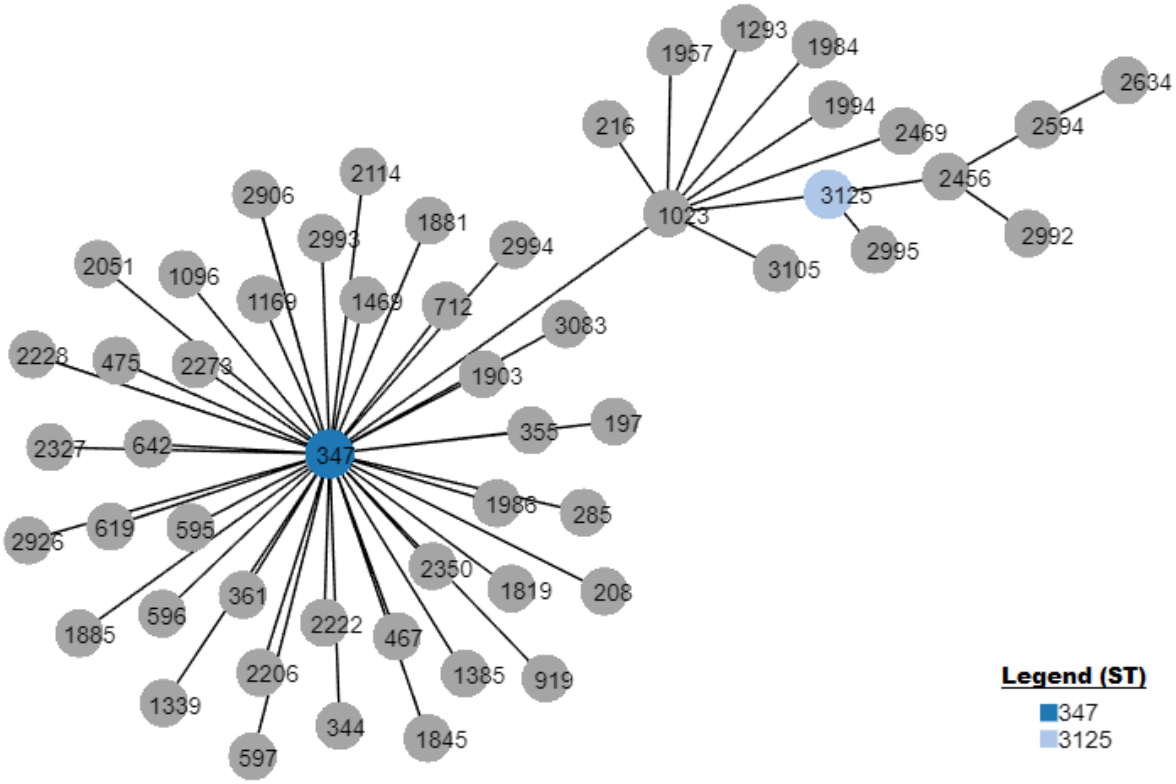

Figure 2 Minimum spanning tree showing *K. pneumoniae* ST3125 arising from the ST347-lineage. Each node represents a distinct ST and each branch represents a single locus variant. ST3125 is a double locus variant of ST347. Branch lengths are not proportional to genetic distance.

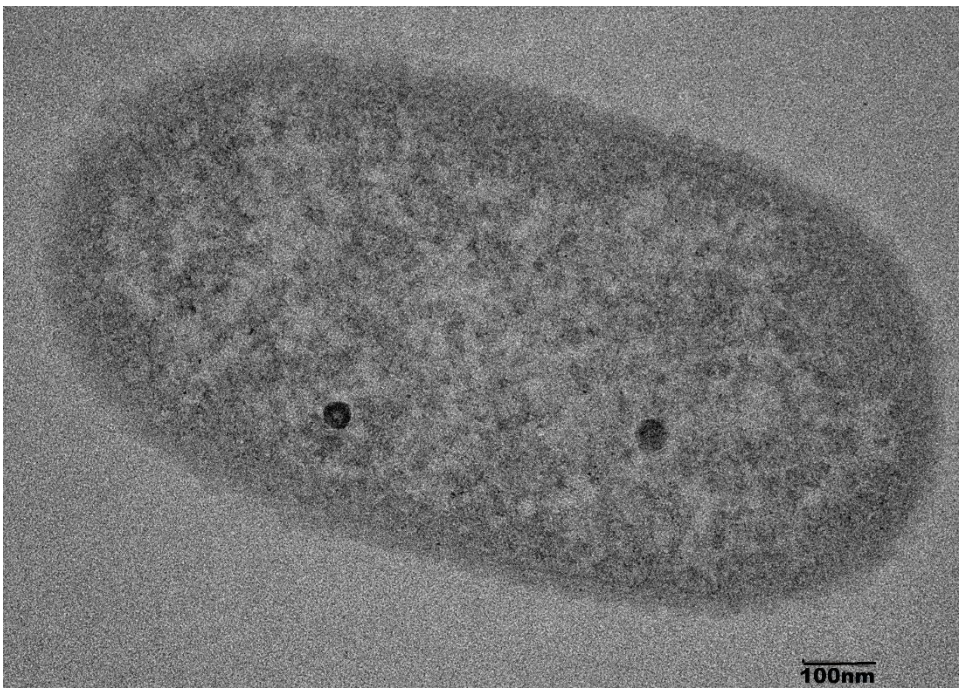

Figure 3 Transmission electron microscopy imaging of *C. gleum* UM-AEU276 at 40,000x magnification.

Analysis of *K. pneumoniae* genetic relationships using minimum spanning trees displayed that the strains were derived from two central founders; ST1440 emerged from ST17 (Figure 1) while ST3125 emerged from ST347 (Figure 2). ST1440 was a double locus variant of ST17 and a single locus variant of ST20 (Figure 1). Multidrug-resistant and hypervirulent ST17 strains have been reported in Africa (Henson et al., 2017), Asia (Jin et al., 2015), Europe (Löhr et al., 2015), Latin America (Pasteran et al., 2012) and North America (Cerqueira et al., 2017), epitomizing their competence for colonization and global spread. In China, ST20 has been reported to cause epidemics in a neonatal unit (Jin et al., 2015) and

ST1440 has been grouped among high-risk clones in Taiwan (Yan et al., 2015). A 3-year outbreak in a medical center involving ST2252, which evolved from ST1440, occurred despite strict infection control measures and ST2252 was also shown to spread carbapenem-resistance to other bacteria species (Kanamori et al., 2017). The novel ST3125 was derived from ST347 and ST1023, previously found in pneumonia patients from Japan (Ito et al., 2015) and from bloodstream infections in Shanghai (Xiao et al., 2017). Interestingly, ST2992, ST2995 and ST2594 human rectal strains which were derived from ST3125, were postulated as human commensal strains from gut microbiota (Gorrie et al., 2018).

Overall, genetic analyses of the *K. pneumoniae* strains from this study revealed that ST1440 (Figure 1) can potentially establish itself as the dominant clone in satellite animal laboratories as it belongs to a lineage which has the propensity for colonization (Pasteran et al., 2012; Jin et al., 2015; Cerqueira et al., 2017). This was evident in the discovery of ST1440 from two different laboratories at different periods. The use of individually ventilated cages (IVC) provided a measure of biocontainment when infected mice were housed with non-infected mice, preventing pathogen transmission to other mice within the same facility (Brielmeier et al., 2006). *K. pneumoniae* UM-AEU320 (ST1440) was cultured from a sentinel mouse housed in an open cage, perhaps exposing it to *K. pneumoniae* contaminated fomite from the environment (Lautenbach et al., 2001). Meanwhile, ST3125 (Figure 2) which has multidrug resistant ancestors (Ito et al., 2015; Xiao et al., 2017) and presumably harmless human commensal descendants (Gorrie et al., 2018), could potentially express hypervirulent phenotypes under antimicrobial selection pressure (Holt et al., 2015).

On the basis of our findings, as well as the effectiveness of the sentinel program, we stress that stringent hygiene practices and rigorous infection control protocols must be vigilantly practiced in any animal laboratory to prevent the colonization and spread of undocumented infectious pathogens. The health and infection status of laboratory rodents have to be monitored closely to ensure that the animals are free of unwanted infections that could jeopardize the interpretation and reliability of animal experiments.

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บทคัดย่อ

Effectiveness of sentinel rodents for surveillance of exposure to undocumented bacterial pathogens in animal research facility

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The use of sentinel animals in animal research facilities is the cornerstone of animal health monitoring for exposure to natural pathogens of laboratory animals. This is because infection with these pathogens produces no overt signs of disease yet the infection may affect the outcome of research utilising these animals. One of the important pathogens includes *Klebsiella pneumoniae*, whose prevalence can be high in laboratory animals. This study sought to document the prevalence of *K. pneumoniae* and other undocumented bacterial pathogens in laboratory rodents for a 3-year period. *K. pneumoniae* and *Chryseobacterium gleum* were isolated from sentinel ICR mice housed in three different satellite animal laboratories, suggesting the effectiveness of the sentinel program. A novel strain of *K. pneumoniae* ST3125 was recovered from the gastrointestinal tract of the ICR mouse. β-lactamase and virulence genes were detected among *K. pneumoniae* and *C. gleum* strains, suggesting the acquisition of these genes from the users of the animal research facilities. Examination of the animal housing environment, feed and water specimens however, returned negative for the presence of *K. pneumoniae* and *C. gleum* suggesting that current hygiene practices were adequate in controlling transmission from the environment. Nevertheless, stringent hygiene practices and infection control protocols have to be applied in animal facilities to prevent the colonization and spread of pathogens capable of distorting experimental results.

คำสำคัญ: *Chryseobacterium gleum* infectious disease *Klebsiella pneumoniae* laboratory animals Malaysia

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