Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

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

Document heading doi:

Isolation and identification of multidrug-resistant *Staphylococcus haemolyticus* from a laboratory-breeding mouse

Fengying Huang[1, 2, 3], Qiuping Meng[1◇], Guanghong Tan[1*], Yonghao Huang[1], Hua Wang[1], Wenli Mei[3], Haofu Dai[3]

[1]*Hainan Provincial Key Laboratory of Tropical Medicine, Hainan Medical College, Haikou 571101, China*
[2]*Agriculture School, Hainan University, Haikou 571101, China.*
[3]*Biological Technology Institute, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, China*

ARTICLE INFO

Article history:
Received 21 February 2011
Received in revised form 11 April 2011
Accepted 15 May 2011
Available online 20 June 2011

Keywords:
16S rRNA
Gene sequences analysis
Staphylococcus haemolyticus
Multidrug resistant

ABSTRACT

Objective: To analysis and identify a bacterium strain isolated from laboratory breeding mouse far away from a hospital. **Methods:** Phenotype of the isolate was investigated by conventional microbiological methods, including Gram-staining, colony morphology, tests for haemolysis, catalase, coagulase, and antimicrobial susceptibility test. The *mec*A and 16S rRNA genes were amplified by the polymerase chain reaction (PCR) and sequenced. The base sequence of the PCR product was compared with known 16S rRNA gene sequences in the GenBank database by phylogenetic analysis and multiple sequence alignment. **Results:** The isolate in this study was a gram positive, coagulase negative, and catalase positive coccus. The isolate was resistant to oxacillin, methicillin, penicillin, ampicillin, cefazolin, ciprofloxacin erythromycin, *et al*. PCR results indicated that the isolate was *mec*A gene positive and its 16S rRNA was 1 465 bp. Phylogenetic analysis of the resultant 16S rRNA indicated the isolate belonged to genus *Saphylococcus*, and multiple sequence alignment showed that the isolate was *Saphylococcus haemolyticus* with only one base difference from the corresponding 16S rRNA deposited in the GenBank. **Conclusions:** 16S rRNA gene sequencing is a suitable technique for non-specialist researchers. Laboratory animals are possible sources of lethal pathogens, and researchers must adapt protective measures when they manipulate animals.

1. Introduction

Bacteria identification in hospital microbiology laboratory is usually done traditionally by isolating the organism and studying it phenotype by means of culture, biochemical methods, and Gram staining[1], which have been accepted as the gold-standard for bacterial identification in clinical microbiology laboratory. However, there are three major drawbacks in these methods of bacterial identification. First, these methods cannot be used for non-cultivable bacteria. Second, there are some specific bacteria whose biochemical characteristics do not fit into the patterns of any known genus and species. Third, it is difficult and too complex for researches who have not received related training to adapt these methods to identify bacteria. Due to the discovery of the polymerase chain reaction (PCR) and DNA sequencing, some bacterial genomes have been sequenced completely[2]. The comparison of the bacterial genomic sequences demonstrated that the 16S ribosomal RNA (rRNA) gene is highly conserved among species of the same genus[3, 4]. Thus, 16S rRNA sequencing has been adapted as a new gold-standard for identification of bacteria. Using this new gold-standard, many non-cultivable bacteria and bacteria with non characteristic biochemical patterns are classified and re-classified into new genera[5-8].

Staphylococcus haemolyticus (S. haemolyticus) is an

*Corresponding author: Guanghong Tan, Hainan Provincial Key Laboratory of Tropical Medicine, Hainan Medical College, Haikou 571101, China.
◇Qiuping Meng, Undergraduate Student of Hainan Medical College, contributed equally to this work.
Tel & Fax: 86-898-66893335
E-mail: scihai@126.com
Foundation project: This work was partially funded by the National Natural Science Foundation of China (No. 30960411) and 973 Program (No. 2010CB534909).
The GenBank accession number for the 16S rRNA sequence of this isolate in this study is JF775574.

opportunistic bacterial pathogen found in the normal skin flora, commonly isolated from the axillae, perineum and inguinal areas from humans[9]. Study results have shown that *S. haemolyticus* is one of the most commonly coagulase-negative *Staphylococcus* species isolated from patients and healthcare workers in hospital environments, which is the major pathogens causing septicaemia, peritonitis, otitis and urinary tract infections[10-13]. Recently, we found some mice in our laboratory were abnormally dead, which is far away from clinic hospital. We thus cultured the blood samples of these mice, and isolated a strain of multidrug-resistant *S. haemolyticus*, which was at last identified by 16S rRNA gene sequencing and multiple sequence alignment.

2. Materials and methods

2.1. Isolation and culture of bacteria

The suspected mouse was anesthetized by phenobarbital sodium (100 mg/kg), and the thoracic cavity was opened in a sterile state. As much as blood sample was collected by directly heard-puncture, and inoculated into a culture bottle containing 10 mL LB liquid media with sodium heparin as an anticoagulant, and cultured at 37 ℃ overnight with strongly shaking. The cultured bacteria were translated into LB agar plate to separate single colony and one colony was chosen for further culture and analysis. After the antibiotic-resistant was determined, the corresponding antibiotic was added into the media as needed for further culture and study.

2.2. Species identification by conventional methods

The isolated single colony was further cultured and identified by conventional methods, including Gram-staining, colony morphology, haemolysis, tests for catalase, and coagulase. The tests were performed according to standard methodology as described by Versalovic *et al*[14].

2.3. Antimicrobial susceptibility test and MIC determination

Antimicrobial susceptibility testing and determination of MIC were performed using the agar diffusion technique as reported by Swenson *et al*[1]. The antimicrobial agents, which were tested at concentrations ranging from 0.016 to 256 mg/mL, included oxacillin, methicillin, penicillin, ampicillin, cefazolin, ciprofloxacin erythromycin, kanamycin, and gentamicin, clindamycin, vancomycin, cotrimoxazole, fusidic acid, rifampicin, and minocycline. For MIC determination, the bacterium inocula were 5×10^4 CFU/spot. The plates were incubated at 35 ℃ for 24 h, and the MIC endpoint was defined as the lowest drug concentration that inhibited all visible growth.

2.4. Extraction of bacterial DNA

Bacterial DNA extraction was done using the procedures modified from a previously published protocol[15, 16]. Briefly, 80 μL of NaOH (0.05M) was added to 20 μL of bacterial cells suspended in distilled water and the mixture was incubated at 60 ℃ for 45 minutes, followed by the addition of 6 μL of Tris/HCl (pH 7.0), achieving a final pH of 8.0. The resultant mixture was diluted 100× and 5 μL of the diluted extract was used as template for PCR amplification.

2.5. Detection of the mecA gene by PCR

Detection of the *mec*A gene by PCR was done as reported by Jain *et al* and Nguyen *et al*[17,18]. Briefly, the forward and reverse primers used were 5′-CATTTTGAGTTCTGCACTA CC-3′ and 5′-GCAATACAATCGCACATACATTAATAG-3′, respectively. The PCR amplification was performed used PCR Amplification Kit (TaKaRa, Dalian, China) in an automated thermal cycler (Biometra, Germany), the reaction mixture (50 μL in total) contained 5 μL extracted DNA, 5 μL 10×PCR buffer, 4 μL dNTP, 1 μL Taq DNA polymerase and 0.5 μM each primer. The amplified product of 967 bp was detected by Goldview nucleic acid staining (Shanghai SBS, China) following 1.3% agarose gel electrophoresis.

2.6. PCR amplification of 16S rRNA and sequencing

PCR amplification of the 16S rRNA gene was done using 16S rDNA Bacterial Identification PCR Kit (TaKaRa, Dalian, China) and according to manufacturers′ instructions in the attached manual. The contents of the kit contained forward and reverse primers, Taq DNA polymerase, buffer and dNTP mixture. A total 50 μL reaction mixture was mixed as done in the amplification of above *mec*A gene. The mixtures were amplified for 30 cycles at 94 ℃ for one minute, 55 ℃ for one minute, and 72 ℃ for two minutes, with a final extension at 72 ℃ for 10 minutes in an automated thermal cycler (Biometra, Germany). DNase I treated distilled water was used as the negative control. An aliquot of 10 μL of each amplified product was electrophoresed in 1.0% (wt/vol) agarose gel. For DNA sequencing, the PCR product was gel purified using the TaKaRa Agarose Gel DNA Purification Kit (TaKaRa, Dalian, China). Both strands of the PCR product were sequenced twice with Applied Biosystems SOLID 3.0 (Life Technologies, USA) using the following primers, Seq forward 5′-GAGCGGATAACAATTTCACACAGG-3′, Seq reverse 5′-CGCCAGGGT TTTCCCAGTCACG AC-3′, Seq internal 5′-CAGCAGCCGCGGTAATAC-3′.

2.7. Phylogenetic and alignment analysis of the 16S rRNA gene sequence

The neighbour-joining method and software MegAlign 5.0 were used to construct phylogenetic tree as reported by Mulet *et al*[19]. The 16S rRNA gene sequences of reference

genera with their GenBank accession numbers included *Escherichia*, *Shigella*, *Salmonella*, *Citrobacter*, *Yersinia*, *Acinetobacter*, *Pseudomonas*, *Legionella*, *Bartonella*, *Ochrobactrum*, *Streptomyces* and *Staphylococcus*. Each genus were listed at least two strains for phylogenetic analysis. In addition, the sequence of the PCR product was compared and aligned with the 16S rRNA gene sequences in the same genus, which were randomly choose from the GenBank database, by multiple sequence alignment using the Clustal W program as reported by Thompson *et al*[20].

3. Results

3.1. General characteristics of the isolate

Colony morphology showed that the colonies were medium size, round, moist, uplift, white, neat edge, and hemolytic. The isolate was Gram positive coccus (Figure 1A), coagulase negative, and catalase positive.

3.2. Antimicrobial susceptibility and mecA genetype

The isolate was resistant to oxacillin, methicillin, penicillin, ampicillin, cefazolin, ciprofloxacin erythromycin, kanamycin, gentamicin, and clindamycin, and were susceptible to vancomycin, cotrimoxazole, fusidic acid, rifampicin, and minocycline as determined by the standard disk diffusion method. All the MIC values of the resistant antibiotics were ≥256 μg/mL, and the MIC values of vancomycin cotrimoxazole, fusidic acid, rifampicin, and minocycline were less than 2 μg/mL. In addition, as showed in Figure 1B, the results of PCR amplification of *mec*A gene showed that the isolate in this study was *mec*A positive, the amplification of the isolate (Figure 1B, lane 1) and positive control bacterium strain (Figure 1B, lane 2) had a 967 bp products, respectively. However, the PCR amplification of the negative control bacterium strain (ATCC 29213) did not have corresponding product (Figure 1B, lane 3).

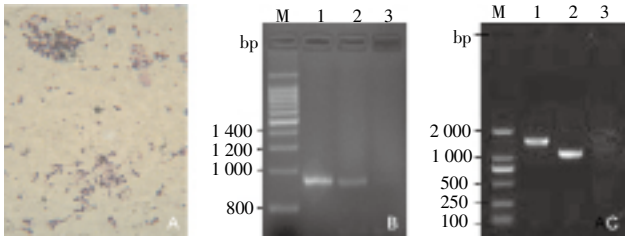

Figure 1. Gram-staining and PCR amplification of *mec*A gene and 16s rRNA.
A: Gram-staining showed positive coccus; B: *mec*A gene positive (lane 1); C: the 16S rRNA of the isolate is about 1 465 bp (lane 1). Lane 2 and 3 in both B and C are corresponding positive and negative controls.

3.3. Phylogenetic analysis of 16S rRNA

Compared with the negative control (Figure 1C, lane 3), PCR amplification of the 16S rRNA gene of the isolate (Figure1C, lane 1) showed a band at 1 465 bp and positive control (Figure 1C, lane 2) showed a band at 1 278 bp. The resultant isolated 16S rRNA was compared with several bacterium genera, including *Escherichia*, *Shigella*, *Salmonella*, *Citrobacter*, *Yersinia*, *Acinetobacter*, *Pseudomonas*, *Legionella*, *Bartonella*, *Ochrobactrum*, *Streptomyces* and *Staphylococcus*. The results of phylogenetic analysis indicated that the isolate in this study belonged to the genus *Staphylococcus* (Figure 2).

3.4. The aligment analysis of 16S rRNA

The base sequences of the isolate was further compared with the corresponding *Staphylococcus* sequences, including *S. haemolyticus* (GenBank accession no. NR036955), *Staphylococcus aureus* (GenBank accession no. L37597), *Staphylococcus epidermidis* (GenBank accession no. HM218280). There was only 1 base difference between the isolate and Staphylococcus haemolyticus, 27 base differences between the isolate and *Staphylococcus aureus*, 25 base differences between the isolate and *Staphylococcus* epidermidis (Figure 2 and 3). The results thus indicated that the isolate in this study was a strain of *S. haemolyticus*.

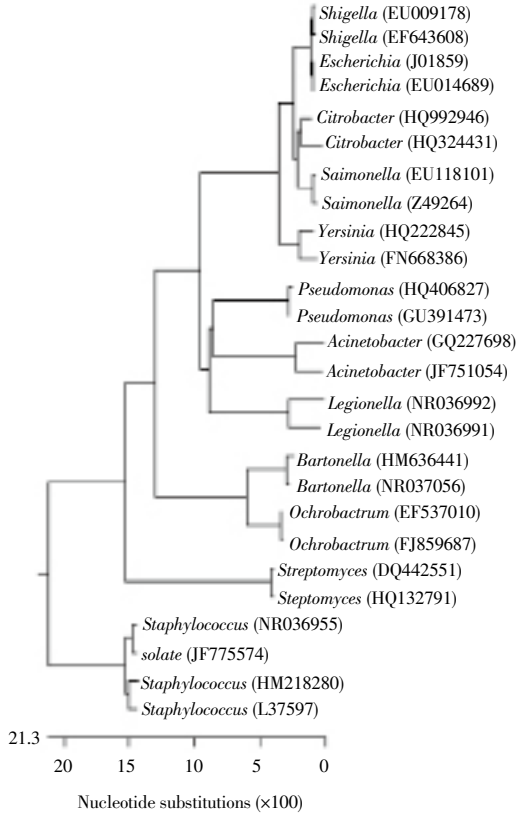

Figure 2. Phylogenetic tree of the 16S rRNAs.
Indicating the isolate in this study belonged to the genus *Staphylococcus*. The numbers in the brackets are GenBank accession numbers.

Figure 3. The aligment of the 16S rRNA sequence of the isolate with the sequences of representative *Staphylococcus*.
The shaded bases represent those in the isolate that are different from the corresponding ones in *S. haemolyticus, Staphylococcus aureus, Staphylococcus epidermidis*.
1: Isolate; 2: *S. haemlyticus*; 3. *Staphylococcus aureus*; 4: *Staphylococcus epidermidis*.

4. Discussion

At present, it was presumed that multidrug resistance mainly exists among *Staphylococci* isolated from medical staff, patients in hospital environments and human breeding animals[21]. However, there is no report on infection of experiment animals, such as mice, in scientific laboratory environments. In this study, we isolated a strain of unknown bacteria from experiment mice. Our results showed that this bacteria strain was Gram positive, coagulase negative, catalase positive and hemolytic coccus. PCR amplification and sequencing of the 16S rRNA indicated that the isolate in this study belong to the genus *S. haemolyticus*, which were concluded from the comparison and alignment of the isolate 16S rRNA with other genus 16S rRNAs in the GenBank.

Laboratory animals, such as mice and rats are widely used in biomedical laboratories. In general, most of the breeders are students who do not know how to identify any unknown bacteria in conventional biochemical methods. Thus, finding an easy method to identify bacteria is highly helpful for such animal breeders. In this study, we used PCR technique to amplify the 16S rRNA and sequencing of unknown bacterium. We further compared and aligned the 16S rRNA sequence with the known sequences in the GenBank, and at last indentified a strain of *S. haemolyticus* from an infected mouse. The use of 16S rRNA gene sequencing to identify unknown bacteria shows the following advantages. First, the turnaround time is shorter than the conventional biochemical methods. Because amplification of the 16S rRNA gene by PCR takes only three to four hours, and sequencing detection of PCR products takes only another few hours, theoretically the identification by the 16S rRNA gene sequencing can be finished within one day. Second, many 16S rRNA sequences have been deposited in GenBank, making it became possible to identify almost any unknown bacterium species in such methods. Third, 16S rRNA gene sequencing can be used not only to common bacteria, but also to other special organisms such as mycobacteria, which can not be identified in most microbiology laboratories because it need other special expertise and equipment, such as gas liquid chromatography. At present, although the cost effectiveness of using 16S rRNA gene sequencing as routine method to identify unknown bacteria remains to be evaluated, our results in this study indicated that application of such techniques to identify unknown bacteria is suitable for students and other non-microbiology specialist researchers.

Staphylococcus, including S*taphylococcus aureus*, *S. haemolyticus, Staphylococcus epidermidis*, is special group of bacteria. Nowadays, methicillin- resistant *Staphylococcus aureus* (MRSA) is an important cause of human nosocomial infections worldwide, and other *Staphylococcus* such as *S. haemolyticus* are seldom reported with methicillin-resistant[22-25]. In our current study, antimicrobial susceptibility tests indicated that the isolate in this study was multidrug resistant. The MIC of oxacillin, methicillin, penicillin, ampicillin, cefazolin, ciprofloxacin erythromycin, kanamycin, gentamicin, and clindamycin were more than 256 μg/mL. In addition, PCR amplification of *mec*A gene indicated that the isolate in this study was *mec*A positive. Methicillin resistance in staphylococci is mediated by the *mec*A gene, encoding the penicillin-binding protein 2a (PBP2a), which has a reduced affinity for the penicillinase resistant penicillins like methicillin and oxacillin and for all other beta-lactam antibiotics[26]. All these data indicated that the isolate in this study is multidrug resistant *S. haemolyticus*.

Multidrug resistant *Staphylococcus* is usually found in patients and medical staff in hospital. Our laboratory locates in an isolated building, which is far away from any hospitals, and our staff seldom contact with hospital staff. Moreover, we never use any antibiotics in our laboratory animals. Thus, it is difficult for us to explain where the multidrug-resistant strain of *S. haemolyticus* come from. Among methicillin-resistant *Staphylococcus, Staphylococcus aureus* are more common and cause more infection in clinical practices[27]. Although, other *Staphylococcus* caused litter damages to human, but they can work as a gene reservoir and translate the multidrug-resistant gene to its counterpart such as *Staphylococcus aureus*. Therefore, our findings in

this study means that laboratory animals are also possible sources of lethal pathogens, and researchers must adapt protective measures when they manipulate any animals in their laboratory.

In summary, we identify a strain of multidrug-resistant *S. haemolyticus* from the mice in our laboratory by 16S rRNA gene sequencing. Our results indicate that identification of unknown bacteria by 6S rRNA gene sequencing is a suitable technique for non-microbiology specialist researchers, such as biomedical students. In addition, our findings also indicate that laboratory animals are also possible sources of lethal pathogens, and researchers must adapt protective measures when they manipulate animals.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Swenson JM, Killgore GE, Tenover FC. Antimicrobial susceptibility testing of *Acinetobacter* spp. by NCCLS broth microdilution and disk diffusion methods. *J Clin Microbiol* 2004; **42**: 5102-5108.

[2] Arnold IC, Zigova Z, Holden M, Lawley TD, Rad R, Dougan G, et al. Comparative whole genome sequence analysis of the carcinogenic bacterial model pathogen *Helicobacter felis*. *Genome Biol Evol* 2011; **3**: 302-308.

[3] Janssen PH. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl Environ Microbiol* 2006; **72**: 1719-1728.

[4] Clarridge JE. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin Microbiol Rev* 2004; **17**: 840-862.

[5] Woo PC, Teng JL, Wu JK, Leung FP, Tse H, Fung AM, et al. Guidelines for interpretation of 16S rRNA gene sequence-based results for identification of medically important aerobic Gram-positive bacteria. *J Med Microbiol* 2009; **58**: 1030-1036.

[6] Nakanaga K, Ishii N, Suzuki K, Tanigawa K, Goto M, Okabe T, et al. *Mycobacterium ulcerans* subsp. shinshuense isolated from a skin ulcer lesion: identification based on 16S rRNA gene sequencing. *J Clin Microbiol* 2007; **45**: 3840-3043.

[7] Gorkiewicz G, Feierl G, Schober C, Dieber F, Kofer J, Zechner R, et al. Species-specific identification of campylobacters by partial 16S rRNA gene sequencing. *J Clin Microbiol* 2003; **41**: 2537-2546.

[8] Conville PS, Witebsky FG. Multiple copies of the 16S rRNA gene in Nocardia nova isolates and implications for sequence-based identification procedures. *J Clin Microbiol* 2005; **43**: 2881-2885.

[9] Takeuchi F, Watanabe S, Baba T, Yuzawa H, Ito T, Morimoto Y, et al. Whole-genome sequencing of *Staphylococcus haemolyticus* uncovers the extreme plasticity of its genome and the evolution of human-colonizing staphylococcal species. *J Bacteriol* 2005; **187**: 7292-7308.

[10]Pi B, Yu M, Chen Y, Yu Y, Li L. Distribution of the ACME-*arc*A gene among meticillin-resistant *Staphylococcus haemolyticus* and identification of a novel ccr allotype in ACME-arcA-positive isolates. *J Med Microbiol* 2009; **58**: 731-736.

[11]Fredheim EG, Klingenberg C, Rohde H, Frankenberger S, Gaustad P, Flaegstad T, et al. Biofilm formation by *Staphylococcus haemolyticus*. *J Clin Microbiol* 2009; **47**: 1172-1180.

[12]Correa JE, De Paulis A, Predari S, Sordelli DO, Jeric PE. First report of *qac*G, *qac*H and *qac*J genes in *Staphylococcus haemolyticus* human clinical isolates. *J Antimicrob Chemother* 2008; **62**: 956-960.

[13]Mack D, Rohde H, Harris LG, Davies AP, Horstkotte MA, Knobloch JK. Biofilm formation in medical device-related infection. *Int J Artif Organs* 2006; **29**: 343-359.

[14]Versalovic J, American Society for Microbiology. *Manual of clinical microbiology*. 10th edition. Washington, DC: ASM Press; 2011.

[15]Woo PC, Leung AS, Leung KW, Yuen KY. Identification of slide coagulase positive, tube coagulase negative *Staphylococcus aureus* by 16S ribosomal RNA gene sequencing. *Mol Pathol* 2001; **54**: 244-247.

[16]Woo PC, Leung PK, Leung KW, Yuen KY. Identification by 16S ribosomal RNA gene sequencing of an *Enterobacteriaceae* species from a bone marrow transplant recipient. *Mol Pathol* 2000; **53**: 211-215.

[17]Jain A, Agarwal J, Bansal S. Prevalence of methicillin-resistant, coagulase-negative staphylococci in neonatal intensive care units: findings from a tertiary care hospital in India. *J Med Microbiol* 2004; **53**: 941-944.

[18]Nguyen DT, Yeh E, Perry S, Luo RF, Pinsky BA, Lee BP. Real-time PCR testing for *mec*A reduces vancomycin usage and length of hospitalization for patients infected with methicillin-sensitive staphylococci. *J Clin Microbiol* 2010; **48**: 785-790.

[19]Mulet M, Gomila M, Gruffaz C, Meyer JM, Palleroni NJ, Lalucat J. Phylogenetic analysis and siderotyping as useful tools in the taxonomy of *Pseudomonas stutzeri*: description of a novel genomovar. *Int J Syst Evol Microbiol* 2008; **58**: 2309-2315.

[20]Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994; **22**: 4673-4680.

[21]French GL. Bactericidal agents in the treatment of MRSA infections-the potential role of daptomycin. *J Antimicrob Chemother* 2006; **58**: 1107-1117.

[22]van Duijkeren E, Box AT, Heck ME, Wannet WJ, Fluit AC. Methicillin-resistant staphylococci isolated from animals. *Vet Microbiol* 2004; **103**: 91-97.

[23] Ramachandran R, Lakshmi R, Kumar RD, Devika K, Rahman F, Wares DF. Fast track method for the identification of multi-drug resistant tuberculosis on direct clinical specimen using combined drug media. *Asian Pac J Trop Dis* 2011; **1**(1): 47-49.

[24] Okoror LE, Eniolorunda TA, Okoror OI. Molecular evolutionary studies of Lassa virus nucleoprotein. *Asian Pac J Trop Dis* 2011; **1**(1): 28-34.

[25] Ravikumar H, Ramachandraswamy N, Puttaraju HP. Molecular strain typing of Wolbachia infection from Indian mosquitoes using wsp gene. *Asian Pac J Trop Dis* 2011; **1**(2): 106-109.

[23]Yasuda R, Kawano J, Onda H, Takagi M, Shimizu A, Anzai T. Methicillin-resistant coagulase-negative staphylococci isolated from healthy horses in Japan. *Am J Vet Res* 2000; **61**: 1451-1455.

[24]Banerjee R, Gretes M, Harlem C, Basuino L, Chambers HF. A *mec*A-negative strain of methicillin-resistant *Staphylococcus aureus* with high-level beta-lactam resistance contains mutations in three genes. *Antimicrob Agents Chemother* 2010; **54**: 4900-4902.