สัตวแพทย์มหานครสาร

JOURNAL OF MAHANAKORN VETERINARY MEDICINE

Available online: www.vet.mut.ac.th/journal_jmvm

การตรวจหาเชื้อแบคทีเรีย Coagulase-positive Staphylococci ที่ดื้อต่อยาในกลุ่มเมทธิซิลินที่แยกจากสุนัขที่เข้ารับการรักษาในโรงพยาบาลสัตว์คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ โดยใช้เทคนิค Duplex PCR

รณภัทร สำเภา[1] และองอาจ เลาหวินิจ[1,#]

[1]คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตบางเขน กรุงเทพฯ 10900

บทคัดย่อ: ในปัจจุบัน Methicillin-resistant coagulase-positive Staphylococci (MRCPS) ในสุนัขเริ่มมีการพบและก่อปัญหามากขึ้น วิธีการตรวจสอบหาเชื้อในกลุ่มนี้ที่ใช้ในห้องปฏิบัติการทั่วไปคือ หาความไวต่อยาปฏิชีวนะ (Drugs susceptibility) โดยการใช้เทคนิค disk diffusion แต่อย่างไรก็ตามวิธีนี้มีข้อจำกัดคือ จำนวนเชื้อที่ใช้เวลาที่ใช้สาหรับการบ่มเชื้ออุณหภูมิ และ pH ของอาหารเลี้ยงเชื้อ ในการศึกษาครั้งนี้จึงใช้เทคนิค Duplex PCR ที่มีข้อดีคือ มีความไวและแม่นยำสูงเข้ามาช่วยยืนยันการดื้อยาของเชื้อ Methicillin-resistant coagulase-positive Staphylococci (MRCPS) โดยหายีนดื้อยา *mec A* และ ยีน *fem B* ซึ่งเป็นยีนที่จำเพาะต่อเชื้อในกลุ่ม coagulase-positive Staphylococci ทำการเก็บตัวอย่างจากสุนัขที่เข้ารับการรักษาในโรงพยาบาลสัตว์ทั้งหมด 115 ตัว โดยวิธี nasal swab และ wound swab สามารถแยกเชื้อกลุ่ม coagulase-positive Staphylococci ได้ทั้งหมด 110 ตัวอย่าง (จากจมูก 60 ตัวอย่าง และแผล 50 ตัวอย่าง) เมื่อนำมาทดสอบ disk diffusion ได้ผลการดื้อต่อยาปฏิชีวนะเมทธิซิลลิน 16 ตัวอย่าง และน่าสงสัยว่าดื้อต่อยาเมทธิซิลลิน (intermediated) 2 ตัวอย่าง หลังจากนั้นนำตัวอย่างไปตรวจโดยเทคนิค duplex PCR เพื่อหายีน *mec A* และ *fem B* พบว่าในกลุ่มที่ดื้อต่อยาเมทธิซิลลิน ให้ผลบวกทั้งหมด 16 ตัวอย่าง สำหรับในกลุ่มที่น่าสงสัยว่าดื้อต่อยาเมทธิซิลลิน (intermediated) ให้ผลบวก1 ตัวอย่าง และผลลบ 1 ตัวอย่าง การศึกษาครั้งนี้แสดงให้เห็นว่ามีการแตกต่างของผลการทดลองทั้ง 2 วิธี ซึ่งเป็นได้ว่าจะมาจากข้อเสียของเทคนิค disk diffusion ในขณะที่การตรวจหา ยีน *mec A* เป็นวิธีที่ได้รับการยอมรับว่าเป็น Gold – Standard สำหรับการตรวจหาเชื้อ methicillin-resistant coagulase-positive Staphylococci (MRCPS) ดังนั้นในการตรวจหาเชื้อในกลุ่ม methicillin-resistant coagulase-positive Staphylococci (MRCPS) จึงมีความจำเป็นที่จะต้องยืนยันโดยใช้เทคนิค duplex PRC

คำสำคัญ: Methicillin-resistant, Coagulase-positive staphylococci (CPS), Dog, Duplex PCR

[#]ผู้รับผิดชอบบทความ

สัตวแพทย์มหานครสาร. 2558. 10(2): 111-118.

E-mail address: fvetonl@ku.ac.th

Detection of Methicillin-resistant Coagulase-positive Staphylococci (MRCPS) Isolated from Dogs Hospitalized to Kasetsart Veterinary Teaching Hospital by Duplex PCR

Ronnapat Sampao[1] and Ongard Lawhavinit[1,#]

[1]Department of Veterinary Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, Bangkean, Bangkok, Thailand, 10900

Abstract: At present Methicillin-resistant coagulase-positive Staphylococci (MRCPS) in dogs became a serious problem. For the principle diagnosis disk diffusion is most often used for drugs susceptibility by disk diffusion but there were some limited conditions, number of bacteria, time for incubated, temperature, and pH. In this study we used duplex PCR technique which confirmed the result from disk diffusion with *mec A* gene and *fem B* detection. 115 samples were collected from nasal swab and wound swab from dogs hospitalized to veterinary hospital. 110 samples were coagulase-positive Staphylococci (nasal swab = 60, wound swab = 50. There were 16 samples positive for methicillin-resistance, 2 were intermediated by disk diffusion but 17 were positive including one of intermediated group and 1 was negative from detection with Duplex PCR. This study shown that there were difference results among 2 techniques which may be some disadvantage of disk diffusion on the other hand Duplex PCR is gold standard for detection methicillin-resistant coagulase-positive Staphylococci **(**MRCPS). So detection for methicillin-resistant coagulase-positive Staphylococci **(**MRCPS) has to use Duplex PCR.

Keywords: Methicillin-resistant, Coagulase-positive Staphylococci **(**CPS), Dog, Duplex PCR

[#]Corresponding author

E-mail address: fvetonl@ku.ac.th

J. Mahanakorn Vet. Med. 2015. 10(2): 111-118.

Introduction

Coagulase-positive staphylococci (CPS), gram-positive cocci bacteria are well-known opportunistic and pathogenic bacteria in both of man and animals. Which cause mild skin infection to severe bacteremia, pneumonia, osteomyelitis, mastitis, scalded skin syndrome and abscesses of the muscle,

urogenital tract and various intra-abdominal organs (Murray *et al.,* 2003; Leonard and Markey, 2008. In particular, methicillin-resistant *Staphylococcus aureus* (MRSA), an important cause of nosocomial infection in humans and dogs. The first report of methicillin resistant MRSA in dogs was report in 1999 in South Korea. *Staphylococcus aureus* More than 80% were found antibiotic resistance, made problem for medical treatment in animal. At present, methicillin-resistant coagulase-positive Staphylococci (MRCPS) has become an important problem in veterinary teaching hospital because MRSA is known to be one of the most prevalent nosocomial pathogens throughout the world and to be capable of causing a wide range of hospital-linked infections (John Hwa Lee, 2003). Other coagulase-positive staphylococci are primarily of relevance for dogs and cats, namely *Staphylococcus intermedius*, *Staphylococcus schleiferi* subsp. *coagulans*, and *Staphylococcus. pseudintermedius*. These species are commensal organisms, but are also a cause of disease (such as pyoderma and otitis externa) in both dogs and cats (Morris *et al.,* 2005; Devriese *et. al.,* 2005). The major of resistance of Methicillin-resistant coagulase-positive Staphylococci (MRCPS) mediated by the *mec A* gene that encodes a modified penicillin-binding protein (PBP), the PBP2a or 2', which shows reduced affinity to the, such as methicillin and oxacillin (van Duijkeren *et al.,* 2004). For detection of MRCPS there are three methods, two methods based on drug susceptibility test are detection of MIC concentration, or disk diffusion test (CLSI, 2009), a standard for detection MRCPS in laboratory which has disadvantages, inoculums size, pH, time and temperature for incubation. The last method is PCR technique which is Gold – Standard for detection MRCPS (Datta *et al.,* 2011) in this study we detected the *fem B* which species-specific for CPS such as *Staphylococcus* or *Staphylococcus. pseudintermedius* (Takashi *et. al.,* 2006; Gunawardena *et al.,* 2012) and the *mec A* gene which encoding methicillin resistant.

Objective of this study was detection of methicillin resistant coagulase-positive Staphylococci (MRCPS) from dogs hospitalized to Kasetsart veterinary teaching hospital, Thailand by Duplex PCR

Materials and Methods

1. Samples Collection and Bacterial Isolation

1.1 Samples collection from dogs

Samples were collected from 110 dogs hospitalized to Kasetsart Veterinary Teaching hospital by nasal swab or wound swab from December 2013 to April 2014

1.2 Bacterial Isolation

The sample swabs were transfer to tryptic soy broth with 10% NaCl and incubated at 37ºC 18-24 hrs for selective enrichment of Staphylococci) Loeffler *et al.,* 2005). Then the culture was streaked on to Blood agar incubated at 37ºC for 18-24 hrs to check for hemolysis and streaked onto Nutrient agar *(*NA*)* for gram straining, catalase test, coagulase test and re-streaked onto Mannitol salt agar for presumptive CPS identification.

2. Methcillin-resistance detection

2.1 Drugs susceptibility test

Methicillin resistant was determined by disk diffusion method using Oxacillin 1 μg according to the guidelines of Clinical and Laboratory Standards Institute (CLSI). Resistance to antimicrobials was determined with inhibition zone < 11 mm *(*CLSI, 2009*)*.

2.2 Duplex PCR for detection of mec A and fem B genes.

DNA extraction

DNA of bacterial was extracted by using "InstaGene™ Matrix" kit (Bio-Rad, USA). Put 1 pure colonies of the CPS in1 ml of sterile distilled water after subjecting each extract to centrifugation for 1 minute at 10,000 rpm. The supernatant was removed and then 200 µl of "InstaGene™ Matrix" kit was added and then incubated at 56°C for 15-30 minutes before spinning on a vortex for 10 seconds and then incubating at around 100°C for 8 minutes. This was followed by a final spin in the Vortex for10 seconds and then centrifuge again at 10,000 g for 2-3 minutes to extract the DNA. DNA concentration was determined by using nano drop

PCR primers

For the *mec A* and *fem B* genes detection using primer *mecA*1 (5-GTA GAA ATG ACT GAA CGT CCG ATAA) and *mecA*2 (5-CCA ATT CCA CAT TGT TTC GGT CTA A), PCR product was 310-bp while the *femB* Gene was detected with the primers *FemB*1 (5-TTA CAG AGTTAA CTG TTA CC) and *FemB*2 (5-ATA CAA ATC CAG CAC GCT CT), PCR product was 651 bp *(* Jonas *et al.,* 2002; Takashi *et. al.,* 2006; Gunawardena *et al.,* 2012*)*

Duplex PCR

The duplex PCR for detection of MRSA was performed essentially as described previously *(*Towner *et al.,* 1998*)*. The PCR cycling conditions were as follows: initial denaturation at 94°C for 4 min, followed by 30 cycles of 45 s at 94°C, 45 s at 50°C, and 60 s at 72°C, with a final extension step at 72°C for 2 min. PCR products were analyzed

by electrophoresis in 1 Tris-Boric-EDTA on a 0.8% agarose gel stained with ethidium bromide and amplicons were visualized using a UV light box.

Results

Of total 115 dogs were isolated 110 as CPS by phenotypic identification (β-hemolysis on blood agar, gram straining, catalase, coagulase test and Mannitol salt agar) 60 from nasal swab and 45 from wound swab. 16 isolates were MRSA (inhibition zone >13 mm by CLSI, 2009), 2 were intermediated by disk diffusion method (inhibition zone 11-12 mm by CLSI, 2009) (Table1). But after detection by duplex PCR 17 isolates of resistant group including one of intermediated were positive to MRCPS, but only 1 isolate of intermediated group was negative to MRCPS by duplex PCR (Fig1, Tab2).

Discussions and Conclusion

The identification of methicillin-resistant MRCPS in laboratory is sometimes maybe has some complication because of many factors. In this study 115samples were collected from dogs and 110 were isolated as CPS by phenotypic identification and restreak onto blood agar including on manitol salt agar. 16 isolates were MRCPS and 2 were intermediated by disk diffusion technique (Table1) (CLSI, 2009). When detection by duplex PCR 17 isolates including one of intermediated group from above were MRCPS and 1 isolate from intermediated group was negative (Fig1). As results there were difference among disk diffusion and Duplex PCR. While gold-standard for MRCSP detection (ex. MRSA) is mec A detection as described previously (Datta *et al*., 2011). Because of limited condition such as number of bacteria, temperature and pH in media of disk diffusion method (Skove, 2006; Hartman and Tomasz., 1984; Madiraju *et al*., 1987; Sierra-Madero *et al*., 1988b; Ataee *et al*., 2012) maybe effected on result as we can saw from 2 suspected samples but when we brought that sample into Duplex PCR which second gene was fem B CoPS specific gene (Takashi et. al., 2006; Gunawardena *et al*., 2012). The result show that one of suspected was MRCPS positive and the other one was MRCPS negative (Fig1). According the results from disk diffusion method there are many disadvantage and limited condition as described above.

Table 1 Show result from detection of MRCPS by disc diffusion technique

Sources	Number of Samples	
	Resistance	Intermediated
Nasal swab	12	2
Wound swab	6	

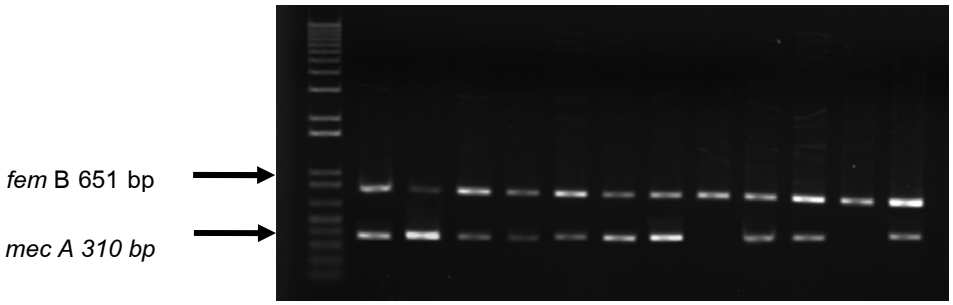

Fig.1 Show Duplex PCR for detection of methicillin-resistant coagulase-positive Staphylococci *(MRCPS)* lane M = 100 bp marker, lane 1-7, 9 = MRSA, lane 8 = suspected sample from disc diffusion, Lane 10 = suspected sample from disc diffusion, Lane 11 = negative control and Lane 12 = positive control (*Staphylococcus aureus* MRSA strain DMST 20651)

Table2 Comparison between disc-diffusion method and Duplex PCR

Techniques	**Number of MRCPS Positive sample**
Oxacillin-disc diffusion	16
Duplex PCR	17

This study show that Duplex PCR which detected mec A gene and fem B gene is better than disk-diffusion method from many reasons (Sensitivity, accuracy and spend less time) and can be solved problem in disk diffusion method. Therefore, the best option method for detection methicillin-resistant coagulase-positive Staphylococci (MRCPS) is Duplex PCR.

Acknowledgements

The authors would like thank you all of staff in department of Veterinary microbiology and immunology for corporate through this study.

References

Ataee, R. A., Mehrabi, T.A., Seyed, M.J. H., Khadijeh, M., and Mahdi G. Z. 2012. A Method for Antibiotic Susceptibility Testing: Applicable and Accurate. *J. Microbiol*. 5: 341-345.

Clinical and Laboratory Standards Institute (CLSI). 2009. Performance Standards for Antimicrobial Susceptibility Testing: Nineteenth Informational Supplement. Approved standard, 10th ed. *M02-A10 Clinical and Laboratory Standards Institute (CLSI)*, Wayne, P.A. Prevalence of methicillin-resistant *S.aureus* among staff and pets in a small animal referral hospital in the UK. *J. Antimicrob. Chemother*. 56: 692–697.

Jonas, D., Speck, M., Daschner F. D. and Grundmann, H. 2002. Rapid PCR-Based identification of Methicillin-Resistant *Staphylococcus aureus* from Screening Swabs. *J. Clin. Microb.* 40: 1821-1823.

Datta, P., Gulati, N., Singla, N., Vasdeva, H. R. and Bala, K. 2011. Evaluation of various methods for the detection of meticillin resistant *Staphylococcus aureus* strains and susceptibility patterns. *J. Med. Microb*. 60: 1613-1616.

Devriese, L.A., Vancanneyt, M., Baele, M., Vaneechoutte, M., De Graef, E., Snauwaert, C., Cleenwerck, I., Dawyndt, P., Swings, J., Decostere. A. and Haesebrouck, F. 2005. *Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals. *Int. J. Syst. Evol. Microbiol*. 55: 1569–1573.

Hartman, B. J. and Tomasz, A. 1984. Low-affinity penicillin- binding-protein associated with b-lactam resistance in *Staphylococcus aureus*. *J. Bacteriol*. 158: 513–516.

John, H. L. 2003. Methicillin (Oxacillin)-Resistant *Staphylococcus aureus* Strains Isolated from Major Food Animals and Their Potential Transmission to Humans. *Appl. Environ. Microb.* 69: 6489-6494.

Towner, K. J., Talbot D. C. S., Curran R., Webster C. A. and Humphreys H. 1998. Development and evaluation of a PCR-based immunoassay for the rapid detection of methicillin-resistant *Staphylococcus aureus*. *J. Med. Microb.* 47: 607-613.

Leonard, F.C. and Markey, B.K. 2008. Meticillin-resistant *S. aureus* in animals: A review. *The Vet J.* 175: 27-36.

Madiraju, M.V.V.S., Brunner, D.P. and Wilkinson., B.J. 1987. Effects of temperature, NaCl, and methicillin on penicillin-binding proteins, growth, peptidoglycan synthesis, and autolysis in MRSA. *Antimicrob. Agents. Chemother*. 31: 1727-1733.

Morris D.O., Rook K.A., Shofer F.S., Rankin S.C. 2006. Screening of *Staphylococcus aureus*, *Staphylococcus intermedius*, and *Staphylococcus schleiferi* isolates obtained from small companion animals for antimicrobial resistance: A retrospective review of 749 isolates (2003–04). *Vet. Dermatol*. 17: 332–337.

Morris, D.O., Mauldin, E.A., O'Shea, K., Shofer, F.S. and Rankin, S.C. 2006. Clinical, microbiological, and molecular characterization of methicillin-resistant *Staphylococcus aureus* infections of cats. *Am. J. Vet. Res.* 67: 1421–1425.

Murray, P.R., Baron, E.J., Jorgensen, J.H., Pfaller, M. A. and Yolken, R.H. 2003. *Manual of Clinical Microbiology, 8th edn.* Washington, DC: American Society for Microbiology.

Sierra-Madero, J.G., Knapp, C., Karaffa, C. and Washington, J.A. 1988. Role of beta-lactamase and different testing conditions in oxacillin-border line-susceptible Staphylococci. *Antimicrob. agents chemother.* 32: 1754-1757.

Skov, R., Smyth, R., Larsen, A. R., Bolmstrom, A., Karlsson, A., Mills, K., Frimodt-Moller, N. and Kahlmeter, G. 2006. Phenotypic detection of methicillin resistance in *Staphylococcus aureus* by disk diffusion testing and Etest on Mueller-Hinton agar. *J. Clin. Microbiol*. 44: 4395–4399.

Takashi, S., Ken, K., Yoshikazu, T., Namiko, T., Shinichi, K. and Keiichi H. 2006. Methicillin-Resistant *Staphylococcus pseudintermedius* in a Veterinary Teaching Hospital. *J. Clin. Microb*. 45: 1118-1125.

Van Duijkeren, E., Box, E., Heck, A.T.A., Wannet, M.E.O.C. and Fluit, A.C. 2004. Methicillin-resistant Staphylococci isolated from animals. *Vet. Microb*. 103: 91-97.