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## Isolation of *Actinobacillus pleuropneumoniae* Serotype 15-like Strain from a Porcine Tonsil in Thailand: A Case Report

Walaiporn Tonpitak

### *Abstract*

An isolate of *Actinobacillus (A.) pleuropneumoniae* from porcine tonsil samples was identified as serotype 15-like strain, which was previously isolated in Australia, North America and Japan. It was characterized by biochemical testing performed for *Actinobacillus* sp. and confirmed as *A. pleuropneumoniae* by PCR and nucleotide sequencing of 16s rDNA. The isolate contains *apxIBD*, *apxIICA*, *apxIIIBD*, *apxIIICA* and *apxIVA* gene. The result of PCR-REA of *apxIVA* method was identical as *A. pleuropneumoniae* serotype 15 strain HS143 reference strain. Therefore this isolate is proposed as *A. pleuropneumoniae* serotype 15-like strain and the first report of *A. pleuropneumoniae* serotype 15-like strain in Thailand. This finding is important for epidemiological investigations of *A. pleuropneumoniae* infections and it may support the selection of an appropriate vaccine formulation.

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**Keywords:** *Actinobacillus pleuropneumoniae*, porcine, serotype 15, Thailand

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## บทคัดย่อ

การแยกเชื้อ แอกติโนบาซิลลัส พลูโรนิวโมนีอี สายพันธุ์ที่มีคุณลักษณะคล้ายคลึงกับซีโรไทป์ 15 จากเนื้อเยื่อทอนซิลสุกรในประเทศไทย-รายงานสัตว์ป่วย

วัลย์พร ตนพิทักษ์

แอกติโนบาซิลลัส พลูโรนิวโมนีอี สายพันธุ์ที่รายงานนี้มีคุณลักษณะคล้ายคลึงกับซีโรไทป์ 15 แยกได้จากเนื้อเยื่อทอนซิลสุกร ซึ่งซีโรไทป์นี้มีรายงานพบในประเทศออสเตรเลีย ทวีปอเมริกาเหนือ และญี่ปุ่น โดยได้ทำการทดสอบคุณสมบัติทางชีวเคมีเพื่อพิสูจน์ว่าเป็นแบคทีเรีย แอกติโนบาซิลลัส สปีชีส์ และยืนยันว่าเป็นแบคทีเรีย แอกติโนบาซิลลัส พลูโรนิวโมนีอี โดยวิธีปฏิกิริยาลูกโซ่โพลีเมอร์เรสและตรวจหาลำดับกรดนิวคลีอิกของ 16เอส ดีเอ็นเอไรโบโซม แบคทีเรียที่แยกได้นี้มียีน *เอพีเอ็กซ์1* *เอพีเอ็กซ์2* *เอพีเอ็กซ์3* *เอพีเอ็กซ์3ซี* *เอพีเอ็กซ์3ซี* *เอพีเอ็กซ์4* ผลการตรวจวิเคราะห์ยีน *เอพีเอ็กซ์4* ด้วยวิธีการตัดผลิตภัณฑ์จากปฏิกิริยาลูกโซ่โพลีเมอร์เรสด้วยเอนไซม์จำเพาะพบว่าให้ผลเช่นเดียวกันกับ แอกติโนบาซิลลัส พลูโรนิวโมนีอี ซีโรไทป์ 15 สายพันธุ์มาตรฐาน HS143 ดังนั้นแบคทีเรียที่แยกได้นี้จึงถูกวินิจฉัยว่าเป็น แอกติโนบาซิลลัส พลูโรนิวโมนีอี สายพันธุ์ที่มีคุณลักษณะคล้ายคลึงกับซีโรไทป์ 15 และเป็นรายงานการพบครั้งแรกในประเทศไทยซึ่งมีความสำคัญในการสอบสวนการระบาดของ แอกติโนบาซิลลัส พลูโรนิวโมนีอี และยังคงมีส่วนช่วยสนับสนุนการเลือกรูปแบบใช้วัคซีนป้องกันโรค

คำสำคัญ: แอกติโนบาซิลลัส พลูโรนิวโมนีอี สุกร ซีโรไทป์ 15 ประเทศไทย

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## Introduction

*Actinobacillus (A.) pleuropneumoniae* is the Gram-negative rod bacteria causing porcine pleuropneumonia and economic loss worldwide to swine industry. It has been classified into two biovars based on growth dependence of nicotinamide adenine dinucleotide (NAD). At least 15 serotypes based on capsular polysaccharide have been defined that vary in geographic distribution. Serotype 1, 5 and 7 are most prevalent in the United States. In Canada, serotype 1, 3 and 5 are isolated frequently, while serotype 1, 2, 5, 7 and 9 are important in Europe (Fenwick and Henry, 1994). In Thailand, serotypes 1 to 7 were isolated (Neramitmansuk et al., 1990; Sthitmatee et al., 2003; Tonpitak, 2009). However, antibody against to *A. pleuropneumoniae* serotypes 1 to 9, 11 and 12 were also detected (Assavacheep et al., 2003; Laohasinnarong et al., 2005). The more recent reported serotype 15 has been found in Australia, North America and Japan respectively but no previous report in Thailand (Blackall et al., 2002; Broes et al., 2007; Koyama et al., 2007). In carrier pigs the pathogens could be isolated from tonsils and, less frequently, from lungs and nasal cavities, and latently infected pigs frequently do not develop a detectable humoral immune response (Gram et al., 2000; Chiers et al., 2002). Therefore, the demonstration of viable *A. pleuropneumoniae* by bacteriological culture from tonsils is still the technique used in many laboratories

(Chiers et al, 2001; Gottschalk et al., 2003). The mechanism of protective immunity to pleuropneumonia caused by *A. pleuropneumoniae* is not completely clear (Rycroft and Garside, 2000). Humoral immunity is considered to be of key importance in the host's protection against *A. pleuropneumoniae*, pigs surviving infection have a protective immunity only to the homologous serotype whereas heterologous serotype protection is variable (Devenish et al., 1990; Bosse et al., 1992; Crujisen et al., 1995). Therefore, the knowledge of serotype distribution as determined by epidemiological monitoring could support in selection of appropriate vaccine for efficient prevention of pleuropneumonia in the respective regions. The purpose of this study is to report the isolation of *A. pleuropneumoniae* serotype 15-like strain that has not been reported in Thailand and could be support the epidemiology of *A. pleuropneumoniae* in Thailand.

## Materials and Methods

**Bacterial strains:** The reference strains of *A. pleuropneumoniae* serotype 1-15 used in this study are described in Table 1.

**Cultivations of samples and identification:** The *A. pleuropneumoniae* isolate was obtained from the tonsil of pig sent to a slaughter house in the Chachoengsao

province. Tonsil samples were processed as described in a previous study (Tonpitak et al., 2007). Briefly, tonsils samples were chopped up with sterile scissor, suspended in phosphate buffer saline (PBS) pH7.2 and vortexed for 30 sec, 100 µl of the suspension were inoculated on MacConkey agar and on a selective agar consisting of Columbia agar supplemented with 5% sheep blood, 3% horse serum, 0.07% NAD, lincomycin (1 µg/ml) and bacitracin (100 µg/ml). The concentration of agar in this medium is 3% agar in order to inhibit *Proteus* spp. that may be present in the samples. Inoculated media were cultured at 37°C and 5% CO<sub>2</sub> for 24 hrs. NAD-dependence and CAMP test performance were investigated on Columbia agar supplemented with 5% sheep blood using *Staphylococcus intermedius* as nursing strain. Complete biochemical tests were performed as previous described by Gottschalk et al. (2003). Briefly, the bacterial strains were cultured on PPLO agar supplemented with 0.01% NAD and finally biochemical tests were performed.

Table 1. *A. pleuropneumoniae* reference strains used in this study

| Serotype | Strain     | Source and reference   |
|----------|------------|--|
| 1        | ATCC 27088 |  |
| 2        | ATCC 27089 |  |
| 3        | ATCC 27090 | American Type Culture Collection   |
| 4        | ATCC 33378 |  |
| 5A       | ATCC 33377 |  |
| 5B       | L20        | Sakpuaram T. (Kasetsart University, Kamphaengsaen Campus, Nakhon Pathom, Thailand) |
| 6        | Fem $\phi$ |  |
| 7        | WF83       | Rosendal S. (University of Guelph, Ontario, Canada)                                |
| 8        | 405        |  |
| 9        | CVJ13261   |  |
| 10       | D13039     | Nielsen R. (State Veterinary Serum Laboratory, Copenhagen, Denmark)                |
| 11       | 56153      |  |
| 12       | 8329       |  |
| 13       | N273       | Angen O. (Institute of Food and Veterinary Research, Copenhagen, Denmark)          |
| 14       | 3906       |  |
| 15       | HS143      |  |

**Genotyping:** In order to confirm that the isolate is not "*Actinobacillus porcitonisillarum*", whose biochemical activities are similar to *A. pleuropneumoniae*, the duplex PCR based on *omlA* gene and intergenic region of *aspC- apxIIC* gene were performed. For the *omlA* gene amplification, the primer LPF and LPR resulting the expected PCR product approximately 950 bp found in *A. pleuropneumoniae* were used. For the detection of intergenic region of *aspC- apxIIC* gene, the primers ASPC-F3/APXIIIC-R3, which amplify the expected 496 bp PCR product found in "*A. porcitonisillarum*" were used (Tonpitak et al., 2007).

Briefly, the 25 µl of PCR reaction mix contained 1 µl of DNA template and composed of 2 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.4 µM of each LPF/LPR and ASPC-F3/APXIIIC-R3 primer and 1 U Taq DNA polymerase. PCR conditions included an initial denaturation step (95°C for 5 min), followed by 30 cycles of 95°C 30 sec, 60°C 30sec, 72°C 1 min and a final extension step at 72°C for 10 min. The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel in 1xTAE buffer and stained with 0.5 µg/ml ethidium bromide.

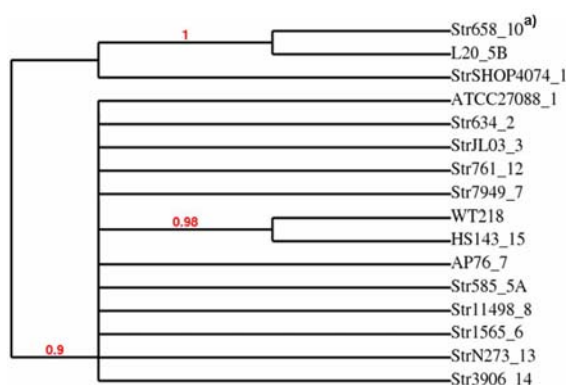
The 16s rDNA were performed by PCR in 20 µl reactions. One microliter of DNA template and primers 27F and 1492R were used. PCR reactions were performed with 35 amplification cycles at 94°C 45 sec, 55°C 1 min and 72°C for 1 min. The PCR product were purified by using Montage® PCR product cleaning kit (Millipore, MA, USA) and the sequenced by MacroGen Inc., Seoul, Korea.

In order to identify the serotype of the isolation, the combination of two PCR reactions reported by Tonpitak (2009) was performed. Furthermore, the PCR restriction analysis (PCR-REA) reported by Turni and Blackall (2007) with some alterations was performed. Briefly, the 50 µl of PCR reactions contained 1 µl of the DNA template and was composed of 60 mM Tris-SO<sub>4</sub> (pH 8.9) 180 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.2 mM dNTP (Promega, MA, USA), 1 U of Platinum® Taq DNA polymerase High Fidelity (Invitrogen, CA, USA), 0.2 µM of each primer of APIVAF and APXIVAR. The PCR conditions included initiation denaturation step at 94°C 2 min, followed by 35 cycles of 94°C 45 sec, 58°C 45 sec, 68°C 6 min and final extension step at 68°C 10 min. Then the 20 µl of PCR products were digested by 6 U of *HhaI* or 10 U of *HpaII* (NEB, MA, USA) at 37°C for 2 hrs. The result was visualized and captured using Gel Doc 2000® system (Bio Rad, CA, USA).

## Results and Discussion

Gram-negative isolates as small, transparent, hemolytic and NAD-dependent colonies showing a positive CAMP- and urease-test and no growth on MacConkey agar were recognised as *Actinobacillus* species. *A. pleuropneumoniae* was confirmed by additional biochemical tests as described by Gottschalk et al. (2003) as well as by duplex PCR and 16S rDNA sequencing as described in previous study (Tonpitak et al., 2007). The isolate *A. pleuropneumoniae*, designated as WT218, was glucose, galactose, mannose, fructose, xylose, sucrose, maltose, mannitol positive and reacted negative to arabinose, lactose, trehalose, sorbitol, inositol, dulcitol, raffinose and salicin. The duplex PCR products found that the isolate WT218 had an outer membrane lipoprotein (*omlA*) gene and did not have the *aspC-apxIIC* intergenic region as well as all the *A. pleuropneumoniae* reference serotype 1 to 15; therefore the isolate WT218 was identified as *A. pleuropneumoniae* (data not shown). The 16S rDNA of isolate WT218 was

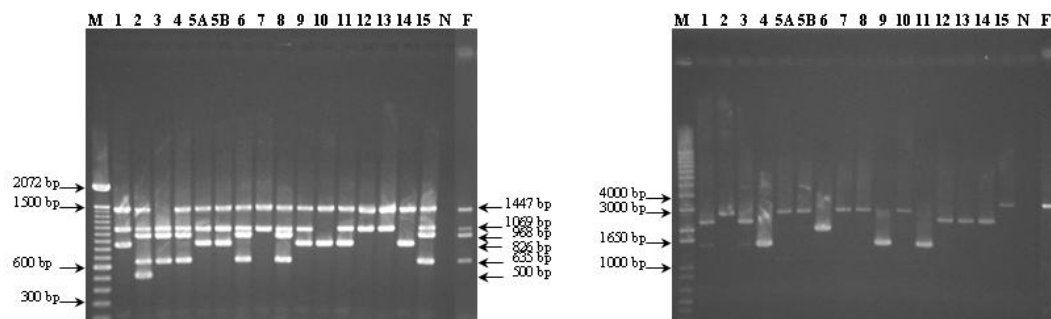
sequenced and Gen-Bank analyses by Blast (<http://www.ncbi.nlm.nih.gov/BLAST>) revealed 99% homology to 16s rDNA of *A. pleuropneumoniae* sequences of strain shop 4074 serotype 1 (D30030), strain 634 serotype 2 (AF033058), strain JL03 serotype 3 (CP000687), strain 585 serotype 5A (AF033060), strain L20 serotype 5B (CP000569), strain 1565 serotype 6 (AF033060), strain 7949 (AF033060) and strain AP76 (CP001091) serotype 7, strain 11498 serotype 8 (AF033060), strain 761 serotype 12 (AF033060), strain 658 serotype 10 (AF033059), strain N273 serotype 13 (AF302255), strain 3906 serotype 14 (AF302256) and strain HS143 serotype 15 (AY01742) and 98% homology to 16s rDNA of *A. pleuropneumoniae* strain ATCC27088 serotype 1 (M75074), which were available in GenBank. Furthermore the phylogeny analysis based on the sequences of 16s rDNA using TreeDyn program (Dereeper et al., 2008) showed that the isolate WT218 is most closely related to the reference strain of serotype 15 (Figure 1).



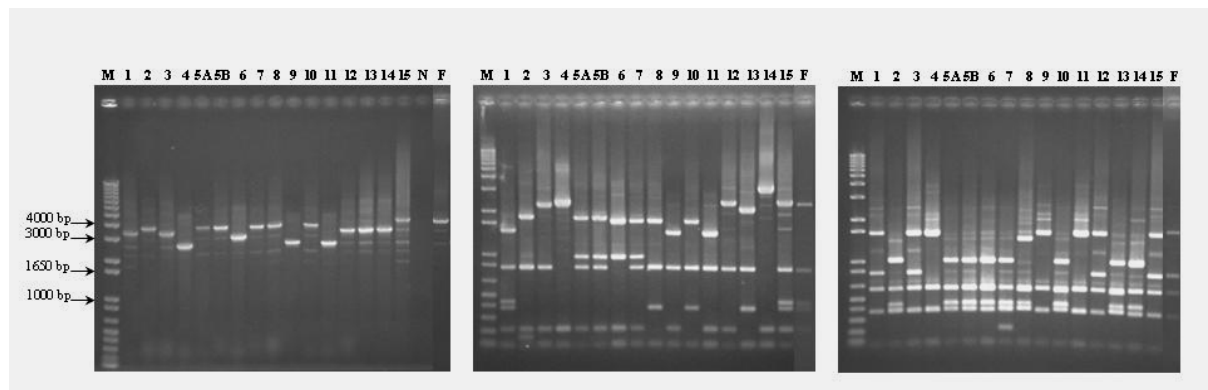
**Figure 1** Phylogeny of the isolate WT218 and *A. pleuropneumoniae* various serotypes, which were available in GenBank, based on analysis of 16s rDNA gene sequences. The sequences of strain 658 serotype 10 (AF033059), strain L20 serotype 5B (CP000569), strain shop 4074 serotype 1 (D30030), strain ATCC07088 serotype 1 (M75074), strain 634 serotype 2 (AF033058), strain JL03 serotype 3

(CP000687), strain 761 serotype 12 (AF033060), strain 7949 serotype 7 (AF033060), strain HS143 serotype 15 (AY01742), strain AP76 serotype 7 (CP001091), strain 585 serotype 5A (AF033060), strain 11498 serotype 8 (AF033060), strain 1565 serotype 6 (AF033060), strain 273 serotype 13 (AF302255) and strain 3906 serotype 14 (AF302256) were obtained from the GenBank database. a) name of strain\_serotype.

The isolate WT218 has *apxIBD*, *apxICA*, *apxIIBD* and *apxIIICA* as serotypes 2, 4, 6 and 8 (Figure 2 left). The PCR product of the *apxIVA* PCR was approximately 3200 bp in size which was equal to that of the reference serotype 15 (Figure 2 right). The *apxIVA* PCR products obtained with primers APIVAF and APXIVAR from the field isolate WT218 and the *A. pleuropneumoniae* serotype 15 reference strain were approximately 4300 bp (Figure 3 left). The results of the *HhaI* digestion of the PCR products of the field isolate WT218 and reference serotype 15 produced bands of 2371, 822, 434, 377 and 208 bp (Figure 3 middle) which were nearly identical to the profile of the serotype 15 reference strain as reported by Turni and Blackall (2007). The results of the *HpaII* digestion of the PCR products of the field isolate WT218 and reference strain of serotype 15 produced bands of 1607, 825, 619 and 377 bp similar to serotype 3 and 12 (Figure 3 right), which were different from the results reported by Turni and Blackall (2007) who did not find the 377 bp fragment of serotype 12 and 15 although the same reference strains were used. It might cause by quantity of the DNA from *apxIVA* PCR product to be used in the restriction reaction was less than in this study. According to all results described above the isolate WT218 was speculated as *A. pleuropneumoniae* serotype 15-like isolate. This is the first report of *A. pleuropneumoniae* serotype 15-like strain isolation in Thailand and it implies that this serotype may be generally present in Thailand. This finding is important for epidemiological investigations of *A. pleuropneumoniae* infections and it may support the selection of an appropriate vaccine formulation.



**Figure 2** Multiplex PCR amplification product of *apxIBD*, *apxICA*, *apxIIBD* and *apxIIICA* gene (left), *apxIV* gene (right) using primers as previously described by Tonpitak 2009; M (left): 100 bp ladder molecular standard (Invitrogen), M (right): 1 kb plus molecular standard (Invitrogen), 1-15: *A. pleuropneumoniae* reference serotype 1-15, N: negative control, F: *A. pleuropneumoniae* isolate WT218.



**Figure 3** PCR amplification product of *apxIVA* gene using primers as previously described by Turni and Blackall (2007) (left), PCR-REA of *apxIVA* gene following digestion with *HhaI* (middle), PCR-REA of *apxIVA* gene following digestion with *HpaII* (right); M: 1 kb plus molecular standard (Invitrogen), 1-15: *A. pleuropneumoniae* reference serotype 1-15, F: *A. pleuropneumoniae* isolate WT218, N: negative control.

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