Isolation of new bacteria farm reared frogs (*Haplobatrachus rugulosus*) in Udon Thani province

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ABSTRACT: The aim of the present study was to isolate bacteria from farm reared frogs in Udon Thani Province, as no previous information exists in this Province despite frog farms are common in this area. Three bacteria were isolated and posteriorly confirmed by sequencing, being *Staphylococcus haemolyticus*, *S. saprolyticus* and *Bacillus siamenis*. We report for first time these three bacteria in frogs. These findings proof the need of bacteriological surveys in farm reared frogs and its possible consequences in its health.

Keywords: *Haplobatrachus rugulosus*, *Staphylococcus haemolyticus*, *Staphylococcus saprolyticus*, *Bacillus siamensis*, farm frog.

Introduction

Isolation of previous clinical reports of farm reared diseased frogs has implicated several bacteria: *Aeromonas hydrophila, Citrobacter freundii, Acinetobacter lwoffii, Flavobacterium* spp., *Pseudomonas* spp., *Staphylococcus epidermidis, Edwardsiella tarda, Proteus* spp., and *Alcaligenes faecalis* as potential pathogens (Mauel et al., 2002). In Thailand, where frog culture is widespread, *Aeromonas hydrophyla* subs. *ranae* was isolated and described from septicemic farmed frogs (*Haplobatrachus rugulosus*) (Huys et al., 2003).

A posterior study in *Rana tigerina* in southern Thailand (Sririkanonda, 2009) reports *Flavobacterium indologenes*, *Staphylococcus sciuri*, *Aeromonas caviae*, *Bacillus brevis, Micrococcus* spp., *Bacillus cereus, Weeksella virosa and Vibrio parahemolytycus.* As presence of bacteria in farm reared frogs is abundant according to literature, the aim of the present study was to isolate bacteria from farm reared farms in Udon Thani Province, as no previous information exists in this Province despite frog farms are common in this area.

Materials and methods

Sample collection

A total of 60 adult reared frogs were randomly collected from 2 farms in Ban Hua Kua and Non Som Boon, Udon Thani, Thailand, in March - May 2014. The frogs were measured (in cm), weighed (in g) and photographed.

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Bacteriological isolation

Five frog organs (heart, kidney, liver, spleen and gall bladder) were collected aseptically. Syringes were used to take blood from heart whereas the other organs were homogenized. Then 0.1 ml of sample solution was diluted with 9 ml of 8.5% NaCl and mixed. 0.1 ml of serial dilutions of 10^{-4} – 10^{-7} were taken to culture in NA agar by spread plate technique. Then, cultured medium were incubated at 37°C for 18-24 hrs.

Bacteriological characterization_by biochemical testing

The incubated plates were examined for morphological characteristics of the cultures representing distinct colonies. Colonies were randomly selected and subcultured to obtain pure isolates on fresh NA plates, then incubated at 37°C for 18-24 hrs. Stock cultures were obtained and carefully labeled, then used for conventional identification using Gram's staining, coagulase test, fermenting mannitol, haemolytic reaction, citrate test, phenol red fermentation and MR-VP test depending on genus of bacteria isolated.

PCR amplification and DNA sequencing analysis

The amplification and sequencing of three isolates (AA09Ki_4, AA10Bl_3 and AB20Ga_1) were performed at the National Center for Genetic Engineering and Biotechnology (BIOTEC). DNA templates were prepared using a Genomic DNA mini kit (Geneaid Biotech Ltd., Taiwan). A PCR product for sequencing 16S rDNA regions was prepared using the following two primers, 20F (5'-GAG TTT GAT CCT GGC TCA G-3') and 1500R (5'-GTT ACC TTG TTA CGA CTT-3'). One hundred µl of a reaction mixture contained 15-20 g of DNA template, 2.0 µmoles of each primer, 2.5 U of *Taq* polymerase, 2.0 mM $MgCl_2$, 0.2 mM dNTP and 1.0 µl of 10x*Taq* buffer (pH 8.8). The PCR amplification was programmed to carry out an initial denaturation step at 94°C for 3 mins, 25 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 2 mins, followed by a final amplification step at 72°C for 3 mins. The PCR product was by 0.8% (w/v) agarose gel electrophoresis and purified. Direct sequencing of the single-band and purified PCR products (ca. 1500 bp) on 16S rDNA was performed in accordance with the *E. coli* numbering system. The nucleotide sequences were using the BioEdit (Biological sequence alignment editor) Program (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Identification of phylogenetic was initially carried out by the BLAST and megaBLAST programs against the database of strain types and published valid prokaryotic nomenclature.

Results

Sampling

Sixty reared frogs representing *Haplobatracus rugulosus* were randomly collected from 2 integrated farms, where farmers grow the frogs in cement ponds, in Udon Thani. The externally clinical signs of abnormality were not found while the internally clinical sign showed white spots in liver and kidney in some frogs.

Bacteriological analysis

A total of 224 colonies of bacteria were isolated from the heart, kidney, liver, spleen and gall bladder of necropsied frogs. Biochemical assays detected round and form in grape-like cluster of

gram-positive bacteria in 109 isolates and short rod of gram-positive bacteria in 115 isolates. Bacteriological analysis of three isolates was shown in **Table 1**.

Table 1 Biochemical assays from target organs (heart, kidney and gall bladder).

Biochemical Tests	AA09Ki_4	AA10Bl_3	AB20Ga_1
Gram staining	Positive	Positive	Positive
Cell shape	Round (grape-like cluster)	Round (grape-like cluster)	Short rod
Coagulase test	+	+	Not tested
Fermenting mannitol	+	+	Not tested
Haemolytic reaction	γ hemolysis	γ hemolysis	Not tested
Citrate test	Not tested	Not tested	+
Phenol red fermentation	Not tested	Not tested	+
MR-VP test	Not tested	Not tested	-/+

PCR amplification and DNA sequencing analysis

The 16S rDNA amplicons were cloned and verified using information retrieved from GenBank databases. Three isolated colonies (AA09Ki_4, AA10Bl_3 and AB20Ga_1) were matched to *Staphylococcus saprophyticus, S. haemolyticus* and *Bacillus siamensis*, respectively, with similar scores of 99.91 (**Table 2**).

Table 2 %Similarity of 16S rDNA compare with closely related species.

Sample number	Species	Strain	Accession	Pairwise Similarity (%)
AA09Ki_4	*Staphylococcus saprophyticus*	ATCC 15305(T)	AP008934	99.91
AA10Bl_3	*Staphylococcus haemolyticus*	ATCC 29970(T)	L37600	99.91
AB20Ga_1	*Bacillus siamensis*	KCTC13613(T)	AJVF01000043	99.91

Discussion and Conclusion

In the review Mauel et al. (2002) none of the bacteria reported in the present study are included. *Bacillus siamenis* was originally isolated from the salted crab (poo-khem) in Thailand (Sumpavapol et al., 2010), being the finding in the present study the first report in frogs.

Staphylococcus saprophyticus was recognized as a cause of urinary tract infections in human (Motwani and Khayr, 2004), our study is the first report in frogs, health effect of this bacterium in frogs is known. The third isolated bacterium in Udon Thani farm reared frogs was *Staphylococcus haemolyticus*, an opportunistic pathogen in man, the presence in frogs could be a cause of disease

being the present study the first report in frogs.

It has been reported (Sririkanonda, 2009) that *Aeromonas caviae* in farm reared *R. tigerina*, as stated previously (Huys et al., 2003) using molecular methods; this species should be transferred to *Aeromonas hydrophila* subsp. *ranae*. As in the present study, molecular methods have to be used complimentary to classical methodological methods to clarify the systematic position of these isolated bacteria and provide quality data.

Farm reared frogs can be infected with several bacteria; in the present study we increase this list of reported species. The finding of these threeve not previously recorded proofs the need of bacteriological surveys in farm reared frogs and the effect of these bacteria in farm production.

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