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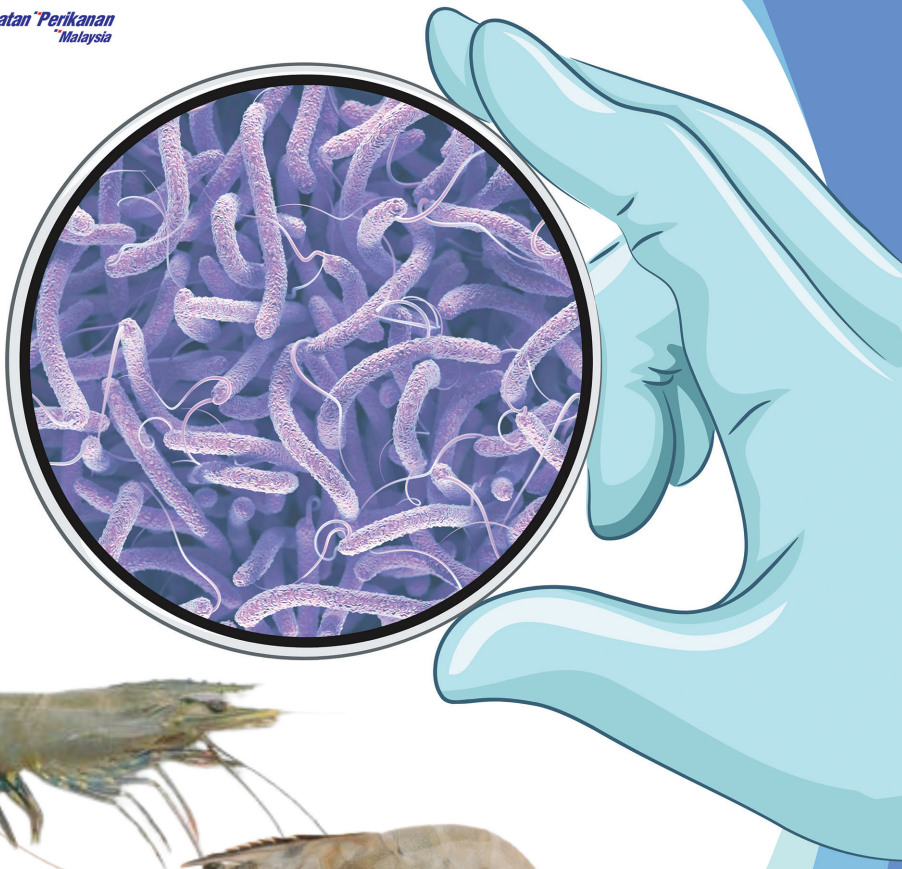

LAPORAN TEKNIKAL

Kajian Early Mortality Syndrome (EMS) /Acute Hepatopancreatic Necrosis Disease (AHPND) Tahun 2011 - 2021

Kua Beng Chu | Padilah Bakar | Iftikhar Ahmad Abdul Rafi

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Laporan Teknikal Kajian EMS/AHPND (Early Mortality Syndrome / Acute Hepatopancreatic Necrosis Disease) Tahun 2011-2021

Laporan Teknikal:

Kajian *Early Mortality Syndrome* (EMS) / *Acute Hepatopancreatic Necrosis Disease* (AHPND)

Tahun 2011 - 2021

Oleh

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PRAKATA

Berkat kerjasama penuh dan komitmen daripada semua penulis yang terlibat, laporan teknikal kajian *Early Mortality Syndrome* (EMS)/*Acute Hepatopancreatic Necrosis Disease* (AHPND) pada tahun 2011-2021 dapat disiapkan. Laporan kajian ini disediakan dengan tujuan agar dapat dijadikan garis panduan kepada pihak-pihak yang terlibat di dalam industri perikanan sama ada di dalam memberi perkhidmatan kepada golongan sasaran dalam menangani permasalahan dari aspek pengurusan ternakan, kawalan dan pencegahan penyakit EMS/AHPND. Laporan ini difokuskan kepada kajian EMS/AHPND di Malaysia yang dijalankan oleh Institut Penyelidikan Malaysia (IPP) melalui Pusat Penyelidikan Kesihatan Ikan Kebangsaan (NaFisH) dan Pusat Penyelidikan Ternakan Krustasea (FRI Pulau Sayak). Kajian bermula apabila berlakunya cetusan wabak penyakit pada ternakan udang marin dengan kematian tinggi dan kerugian yang besar kepada penternak di Malaysia (2011-2014) serta di rantau Asia (2010-2014).

Buku teknikal EMS/AHPND ini dilaporkan secara ringkas tetapi padat dengan menekankan beberapa faktor utama seperti sejarah kes dimana cetusan wabak penyakit EMS/AHPND mula dikesan dan seterusnya senario perebakan penyakit di Malaysia. Kajian dimulakan untuk menentukan jenis penyakit yang menyebabkan peningkatan kes kematian udang di peringkat awal ternakan sehingga disahkan etiologi penyakit dikenali sebagai EMS atau AHPND yang disebabkan oleh bakteria *Vibrio parahaemolyticus* pembawa toksin *PirA/B* AHPND pada 2013. Kajian seterusnya diikuti dengan semakan laporan kes dari makmal diagnosis di NaFisH sehinggalah kepada kajian semula iaitu status pasca jangkitan AHPND di negeri-negeri pengeluar utama udang ternak marin di Malaysia. Selain daripada itu, laporan ini turut menerangkan secara ringkas intipati pelan pihak pengurusan atasan yang melibatkan tindakan dan pelan perancangan kerajaan melalui Jabatan Perikanan Malaysia dalam membendung dan menangani wabak dan perebakan penyakit udang dalam industri akuakultur di Malaysia. Kajian di NaFisH difokuskan kepada kaedah mengenalpasti penyakit AHPND di makmal menggunakan kaedah mikrobiologi, histologi, molekular *Polymerase Chain Reaction* (PCR) dan penjujukan genomik. Salah satu objektif kajian di NaFisH ialah membangunkan satu kaedah pengesanan dan pengesahan AHPND yang cepat, tepat, praktikal dan murah sebagai langkah kawalan di peringkat ladang/kolam. Selepas pengesahan status penyakit AHPND di Malaysia, fasa seterusnya ialah mengenalpasti langkah kawalan atau rawatan yang berfokus kepada penyelidikan dalam meminimakan kerugian akibat penyakit tersebut. Selaras dengan matlamat di atas, kajian di NaFisH juga difokuskan kepada pencarian bahan mesra alam yang boleh digunakan sebagai kawalan/rawatan alternatif menggantikan antibiotik/bahan kimia di dalam mengawal jangkitan penyakit berjangkit.

Kompilasi buku ini diharapkan dapat dijadikan sebagai rujukan kepada pihak-pihak berkepentingan terutamanya penternak udang di Malaysia, kakitangan di Jabatan Perikanan, Pegawai Penyelidik dan pelajar-pelajar di institusi pengajian tinggi. Terima kasih diucapkan kepada penternak di negeri Sabah, Sarawak, Johor, Terengganu, Pahang, Selangor, Perak, Kedah dan Pulau Pinang di atas sokongan dan bantuan sepanjang kajian ini dijalankan.

1.0

LATAR BELAKANG

Komoditi utama dalam industri ternakan udang marin di Malaysia terdiri daripada dua spesis udang ternak iaitu udang harimau (*Penaeus monodon*) dan udang putih (*Penaeus vannamei)*. Sebelum ternakan udang putih, industri ternakan udang tertumpu hanya kepada spesis udang harimau. Namun, industri udang harimau telah terjejas teruk dengan penurunan pengeluaran akibat serangan penyakit bintik putih pada tahun 1996. Situasi ini menyebabkan penternak udang memilih kepada spesis lain seperti udang putih sebagai alternatif ternakan udang marin. Udang putih merupakan spesis eksotik, yang telah dibenarkan untuk diternak secara rasmi di Malaysia bermula pada tahun 2004 bagi mengekalkan pengeluaran udang laut di Malaysia. Pengeluaran udang putih telah meningkat dengan adanya induk *specific pathogen free* (SPF) dan pengenalan kepada strain baru dengan penambahbaikan genetik (*Genetically Improve Strain*) daripada *nucleus breeding centre* (NBC) dari luar negara. Stok benih yang mudah diperolehi merupakan faktor utama menyumbang kepada peningkatan pengeluaran udang putih yang didapati mula melebihi pengeluaran udang harimau pada tahun 2006 (Perangkaan Statistik Perikanan, 2002-2006).

Sejak 2010, kedua spesis udang ini menyumbang kepada pengeluaran utama dalam sektor ternakan air payau. Walau bagaimanapun, kedua-dua spesis mula mengalami penurunan yang ketara pada tahun 2011 berbanding dengan pengeluaran tahun 2010 (Jadual 1) (Perangkaan Statistik Perikanan, 2010-2011). Pengeluaran udang putih terus menurun sehingga tahun 2013 (Rajah 1). Peningkatan pengeluaran mula dikesan pada tahun 2014 dengan nilai harga borong yang tertinggi iaitu RM1.14 bilion melalui pengeluaran sebanyak 57,181 MT berbanding dengan 45,473 MT pada 2013. Namun, penurunan pengeluaran dikesan semula pada tahun berikutnya sehingga 2017. Bermula dari 2018 hingga 2021, pengeluaran udang putih berada dalam julat 35,148 hingga 38,376 MT. Nilai borong udang putih yang direkod sepanjang tahun 2015 hingga 2021 adalah dalam julat RM0.753 hingga RM0.856 bilion.

Situasi yang sama dikesan pada ternakan udang harimau dengan penurunan ketara sebanyak 10,967 MT pada tahun 2011 berbanding dengan pengeluaran tahun 2010 (Rajah 2). Pengeluaran udang harimau dalam julat 4,286 hingga 6,577 MT direkod sepanjang tahun 2012 hingga 2016 dengan harga borong dibawah RM1.64 bilion. Peningkatan mula dikesan bermula dari 2017 dan pengeluaran tertinggi pada 2021 dengan harga borong RM0.539 bilion.

Penurunan kedua spesis udang marin pada 2011 hingga 2014 adalah berpunca daripada pengeluaran udang yang terjejas akibat kematian tinggi di peringkat awal ternakan. Pada masa yang sama, peningkatan kematian udang putih di peringkat awal

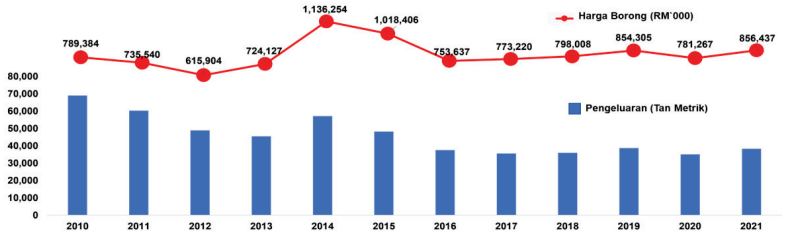
ternakan berumur 15 hari sehingga 30 hari selepas dimasukkan ke kolam ternakan dari peringkat pasca-larva (PL) telah dilaporkan berlaku dimana puncanya tidak dapat dikenalpasti pada waktu tersebut. Penternak merujuk kejadian kematian udang putih di peringkat awal ini sebagai *Early Mortality Syndrome* (EMS) atau 'Pelan-Pelan Mati' (PPM). Pihak Jabatan Perikanan telah menerima aduan dan laporan secara rasmi melalui Bahagian Akuakultur di ibu pejabat pada September 2011. Hasil siasatan di lokasi membawa kepada maklumat awal kejadian kematian tinggi dikesan yang bermula pada akhir Disember 2010, namun tiada laporan aduan rasmi dari penternak dilaporkan kepada Jabatan Perikanan (Rajah 3).

Berdasarkan kepada siasatan tersebut, kejadian EMS telah dikesan akhir pada tahun 2010 dan disahkan secara rasmi pada 2011 (Kua et al., 2016). Kematian udang yang tinggi di peringkat awal usia ternakan ini seterusnya disahkan berpunca dari patogen bakteria *Vibrio parahaemolyticus* yang kemudiannya dikenali sebagai *Acute Hepatopancreatic Necrosis Disease* (AHPND) (Loc Tran et al., 2013). Penyakit ini adalah penyakit baru yang muncul pada tahun 2011 dalam industri udang ternak di Malaysia dan di seluruh dunia. Kematian udang di peringkat awal ternakan turut dilaporkan di China pada 2009, diikuti oleh Vietnam pada 2010 dan Thailand pada 2011 (Lightner et al., 2012; FAO, 2013). Penyakit ini memberi impak kerugian besar kepada penternak kerana kematian udang yang tinggi di peringkat awal usia ternakan. AHPND telah menyebabkan kerugian pengeluaran yang ketara di Mexico, Selatan China dan Asia Tenggara (Shinn et al., 2018). Serangan AHPND mengakibatkan kerosakan organ hepatopankreas secara akut dan menyebabkan kematian udang di peringkat awal ternakan. Kerosakan organ hepatopankreas ini berpunca daripada kehadiran toksin *PirA/B* yang dikeluarkan oleh bakteria *V. parahaemolyticus* (Loc Tran et al., 2013; GAA, 2013; Dangtip et al., 2015)

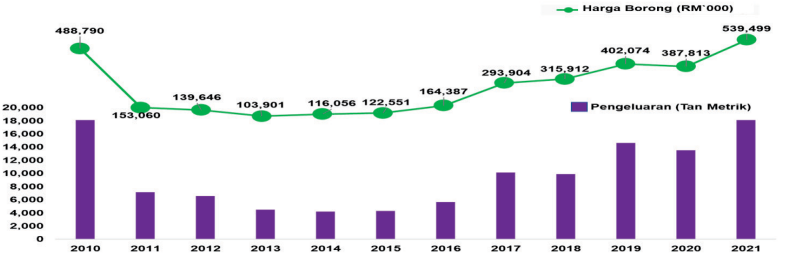
Kajian kematian udang putih oleh Institut Penyelidikan Perikanan melalui Pusat Penyelidikan Kesihatan Ikan Kebangsaan (NaFisH) telah bermula sejak Oktober 2011 berikutan dengan pelaporan kematian tinggi udang di usia awal ternakan. Susulan itu, kajian dimulakan dalam Fasa I iaitu pengesahan kejadian kematian di beberapa lokasi, diikuti dengan Fasa II yang berfokus kepada faktor berkaitan dengan kejadian penyakit tersebut (Jadual 2). Walaupun punca penyebab AHPND telah dikenalpasti pada 2013, namun masih tiada rawatan atau pencegahan keatasnya sehingga tahun 2014. Keadaan ini disebabkan etiologi AHPND adalah bakteria *V. parahaemolyticus* yang biasa dijumpai di persekitaran marin dan kejadian oleh penyakit ini masih menjadi persoalan di kalangan pakar penyakit udang dan negara terlibat pada waktu tersebut. Selepas pengesahan status penyakit AHPND di Malaysia dalam Fasa I, dan II, Fasa seterusnya ialah mengenal pasti langkah kawalan atau rawatan yang berfokus kepada penyelidikan mengenalpasti faktor kejadian utama, langkah kawalan dan rawatan dalam meminimakan kerugian akibat penyakit tersebut.

Jadual 1: Pengeluaran dan harga borong udang ternak marin (udang putih dan udang harimau) dari tahun 2010 hingga 2021

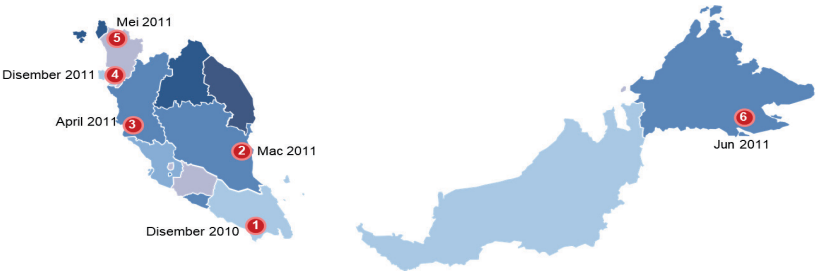
	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021
Pengeluaran (Tan Metrik)	87,203	67,473	55,569	49,957	61,386	52,570	43,248	45,781	45,913	53,400	48,673	56,496
Harga Borong (RM'000)	1,278,174	888,600	755,550	828,028	1,252,310	1,140,957	918,025	1,067,124	1,113,920	1,256,379	1,169,080	1,395,936



Rajah 1: Pengeluaran dan harga borong udang ternak putih dari 2010 hingga 2021.



Rajah 2: Pengeluaran dan harga borong udang harimau dari 2010 hingga 2021.



Rajah 3: Kronologi laporan tidak rasmi kejadian kematian udang putih di peringkat awal ternakan di Malaysia semasa siasatan wabak penyakit pada 2011.

Jadual 2: Fasa kajian AHPND dalam ternakan udang putih

Fasa/ Peruntukan	Objektif	Tempoh	Jenis siasatan dijalankan
I (Tiada peruntukan)	Pengesahan punca kematian akibat penyakit	Sept - Dis. 2011	Sejarah kes, pemerhatian, *anti-clotting time*, bakteriologi, virologi dan histopatologi
II (Tiada peruntukan)	Penentuan faktor berkaitan dengan penyakit	Jan - Mac. 2012	Kajian *cross-sectional* ke atas parameter kimia kualiti air (*dissolved organic carbon / unionized ammonia*), virologi dan pengesanan *Paralytic Shellfish Poison* (PSP)
III (Peruntukan NKEA)	Penentuan lokasi dan status penyakit	Apr - Dis. 2012	Kempen kesedaran EMS, percubaan lapangan, pengesahan PSP toksin sampel (2012) dengan kaedah HPLC & siasatan AHPND di Sabah
IV (Peruntukan NKEA)	Penentuan kaedah rawatan optima bersama penternak	Jan - Apr. 2013	Rawatan fermentasi (Patologi & Bakteriologi)
			Ujian preliminari pemeriksaan smear usus
			Pengesanan toksin PSP sampel dari Perak, Kedah, Sabah & Sarawak
		Apr - Dis. 2013	Pembangunan data awal skor kad usus bagi pengesanan awal AHPND di peringkat ladang
		Mei - Jul. 2013	Eksperimen rawatan percubaan makmal ke atas pasca jangkitan udang EMS dengan aplikasi surfaktan (lipid ester)
		Jul - Dis. 2013	Penentu sah toksin PSP daripada positif sampel (2012/13) menggunakan HPLC
V (Peruntukan NKEA)	Penentuan kaedah pengesanan penyakit hepatopankreas	Jan. 2014 - Dis. 2015	Pembangunan data awal skor kad usus bagi pengesanan awal AHPND dan penyakit mikrospodian di makmal dan lapangan
VI (RMK11)	Validasi kaedah pengesanan penyakit hepatopankreas	Jan. 2016 - Dis. 2017	Ujian cabaran di makmal dan validasi kaedah di lapangan
	Pengenalpastian bahan mesra alam sebagai penyakit hepatoprankeas	Jan. 2018 - Dis. 2020	Saringan bahan mesra alam yang mudah diperolehi termasuk minyak pati komersial
	Pengesahan status penyakit selepas pasca-AHPND	Okt 2018 - Okt 2019	Pemantauan penyakit AHPND di sembilan negeri pengeluar utama udang marin
VII (RMK12)	Aplikasi minyak pati kayu manis sebagai kawalan penyakit hepatoprankeas	Jan 2021 - Dis 2021	Ujian cabaran di makmal dan validasi minyak pati kayu manis sebagai kawalan di lapangan

2.0

JUSTIFIKASI KAJIAN

Akuakultur ternakan udang memainkan peranan penting dalam industri pertanian di Malaysia, terutamanya dalam menyumbang kepada pertumbuhan ekonomi negara (Ghee Thean et al., 2016). Laporan statistik pengeluaran akuakultur menunjukkan pengeluaran udang putih air payau yang mapan dari 2002 to 2010. Walaubagaimanapun, pengeluaran udang menurun sebanyak 13% dan 19% dalam tahun 2011 dan 2012 (Perangkaan Statistik Perikanan, 2002 - 2012). Kematian udang mendadak telah menyebabkan masalah besar kepada penternak dan dikhuatiri boleh membawa kerugian kepada industri akuakultur. Semasa siasatan awal, penternak udang putih melaporkan kejadian kematian mendadak ini telah dialami pada pertengahan tahun 2010 tetapi masih boleh dikawal. Kejadian kematian mendadak didapati menjadi lebih serius selepas pertengahan tahun 2011.

AHPND dikenal pasti di beberapa ladang yang mengalami kematian tinggi pada udang berumur 30 hari selepas dimasukkan ke dalam kolam ternak (DOC 30). Udang yang dijangkiti menunjukkan tanda-tanda klinikal seperti pergerakan yang lemah dan perlahan, kulit lembut, otot berwarna pucat, perut kosong dan hepatopankreas yang pucat. Penyakit in disebabkan oleh bakteria *V. parahaemolyticus* membawa plasmid-enkod binari toksin (*PirA/B*) penyebab utama AHPND. Cetusan penyakit AHPND bersama serangan mikrosporidium parasit *Enterocytozoon Hepatopenaei* (EHP) pada tahun 2010 - 2015 telah menyebabkan kerugian besar kepada penternak udang di seluruh dunia akibat kematian yang tinggi dan terbantut tumbesaran (FAO, 2017). Udang harimau (*P. monodon*), udang putih (*P. vannamei*) and udang putih oriental/chinese (*P. chinensis*) diketahui telah dijangkiti AHPND menyebabkan kematian di antara 40 - 100% selepas 20 - 30 hari udang dimasukkan ke kolam ternak (Lightner et al., 2012).

Sejak tahun 2011-2013, kematian udang putih dari sembilan negeri pengeluaran udang putih iaitu Perak, Pulau Pinang, Kedah, Sarawak, Sabah, Pahang, Johor, Selangor dan Terengganu telah disahkan akibat AHPND. Namun status semasa mengenai kerugian atau impak daripada serangan penyakit ini tidak diketahui dengan jelas memandangkan banyak kes kematian udang putih tidak dilaporkan oleh penternak kepada Jabatan. Ketiadaan kit pengesanan yang cepat dan tepat pada tahun 2013 turut menjejaskan perolehan status sebenar AHPND yang bergantung kepada penemuan bakteria *V. parahaemolyticus* dari organ hepatopankreas udang diikuti dengan pengesahan patogenisiti melalui ujian cabaran bakteria tersebut ke atas udang putih yang sihat. Udang putih yang telah dicabar dengan bakteria ini perlu disahkan melalui kaedah histologi. Ada juga pakar udang yang mengatakan tanda-tanda klinikal seperti kematian tinggi pada udang ternak dan pengesahan patologi AHPND seperti penyingkiran sel epitelium secara akut daripada lapisan dalam dinding tubul organ hepatopankreas di

dalam usus sudah boleh mengesahkan kejadian AHPND. Justeru itu, status AHPND dalam kajian ini ditumpukan kepada kes laporan kematian udang ternakan yang dikesan positif dengan kehadiran bakteria *V. parahaemolyticus* diikuti dengan perubahan patologi AHPND daripada sampel udang berpenyakit.

Langkah awal pencegahan penyakit adalah perlu untuk meminimakan kerugian akibat kematian udang di peringkat permulaan ternakan. Langkah ini perlu dibangunkan melalui kaedah pengesanan yang cepat and awal sebelum udang yang dijangkiti mati. Pada masa tersebut, kaedah pengesanan AHPND adalah melalui kaedah histologi manakala teknik PCR digunakan bermula daripada Februari 2014. Kaedah histologi adalah satu kaedah yang mengambil masa yang panjang disamping memerlukan kepakaran dalam histopatologi analisis, manakala kaedah PCR adalah terlalu mahal untuk penternak kecil. Menurut maklumat persatuan udang Malaysia, 80-90% penternak di Malaysia adalah berskala kecil. Oleh itu, satu kaedah yang senang dan mudah digunakan perlu dibangunkan untuk tujuan pengesanan AHPND sebelum berlakunya kematian. Keputusan kajian Fasa II pada 2012 menunjukkan terdapat sel hepatopankreas di usus udang yang mengalami kematian dan telah disahkan AHPND melalui kaedah histologi. Kaedah pengesanan sel hepatopankreas di usus ini perlu diperincikan untuk tujuan pengesanan awal di peringkat ladang.

Hasil kajian menunjukkan prevalen kehadiran bakteria yang tinggi dikesan daripada organ hepatopankreas udang putih yang telah mengalami kematian di peringkat awal ternakan. Penternak juga melaporkan tumbesaran yang lambat berbanding dengan kadar tumbesaran udang yang tidak pernah mengalami kematian di peringkat awal. Lanjutan daripada penemuan itu, satu kajian lanjutan mengenai bahan yang mesra alam untuk rawatan ke atas udang yang pernah mengalami kematian di peringkat awal ternakan adalah perlu untuk mengurangkan kematian udang secara berterusan.

3.0

OBJEKTIF

Lima objektif telah digariskan dalam kajian AHPND pada udang ternak marin. Objektif tersebut adalah seperti berikut:

I. Menentukan status semasa kejadian tahun 2011 adalah disebabkan penyakit AHPND.

II. Menentukan bilangan kes laporan yang disahkan penyakit AHPND serta kerugiannya.

III. Menentukan status pasca-AHPND di sembilan negeri pengeluar udang ternak marin selepas laporan pertama.

IV. Menentukan bahan mesra alam yang boleh digunakan sebagai kawalan atau rawatan untuk mengurangkan peratus kematian udang akibat AHPND.

V. Membangunkan satu kaedah pengesahan AHPND yang cepat, tepat, praktikal dan murah untuk langkah kawalan di peringkat makmal dan kolam.

4.0

PELAKSANAAN

Pada tahun 2010 hingga 2012, etiologi sebenar kejadian kematian mendadak di peringkat awal ternakan pada ternakan udang putih di Asia masih tidak diketahui. Kematian mendadak ini turut dikenali sebagai Early Mortality Syndrome/Acute Hepatopancreas Necrosis Syndrome (EMS/AHPNS). Ini menyebabkan jurang pengetahuan dan keutamaan kajian R&D perlu diteliti semula termasuklah melihat kepada kewujudan penyakit *cryptic.* Oleh itu, kajian lanjutan R&D perlu diteruskan di dalam mengenalpasti agen penyakit dan toksin hasil daripada siasatan kes diperingkat awal disamping menjawab andaian ke atas kehadiran patogen lain sebagai penyebab kematian atau pencemaran toksin dari persekitaran di Asia. Pada masa yang sama, usaha untuk mengawal atau merawat kejadian penyakit ini turut dilaksanakan. Satu pasukan khas dalam menangani wabak penyakit ini telah ditubuhkan oleh Jabatan Perikanan Malaysia selepas menerima surat rasmi daripada persatuan udang ternak marin Malaysia pada 2011. Kronologi pasukan khas ini merangkumi beberapa siri mesyuarat dan hasil kajian turut dikongsi bersama penternak di seluruh Malaysia dan luar negara (Rajah 4).

Rajah 4: Kronologi tindakan yang telah diambil oleh Jabatan Perikanan dalam menangani wabak penyakit EMS/AHPNS

4.1 Peruntukan

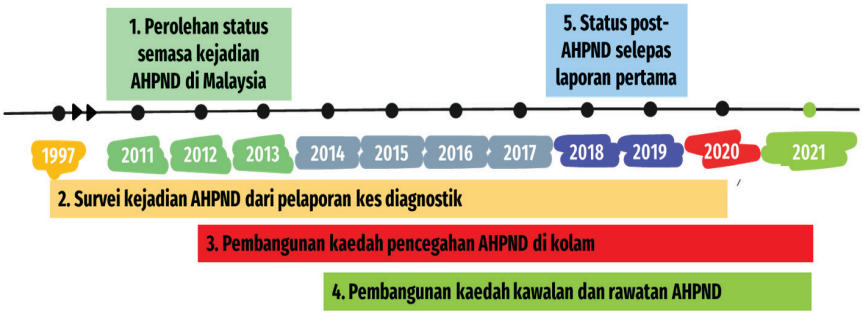
Kajian 2011/2012 lebih tertumpu kepada diagnosis kematian udang putih yang dilaporkan dan kajian lapangan melalui kerjasama dengan penternak. Tiada peruntukan khas untuk kajian EMS/AHPNS pada tahun 2011 dan peruntukan sebanyak RM12,000.00 telah diperolehi pada tahun 2012. Peruntukan sebanyak RM630,000.00 telah dipohon pada tahun 2012 susulan bengkel dan mesyuarat yang dihadiri di Bangkok pada 2012, namun hanya RM200,000.00 diluluskan pada tahun 2013 (Jadual 3). Permohonan kertas kerja dikemuka semula pada tahun 2014 dan melalui dana NKEA, sebanyak RM350,000.00 telah diperolehi secara berfasa untuk tempoh dua tahun (2014/2015). Perolehan dana sebanyak RM150,000.00 di bawah Rancangan Malaysia Ke-11 (RMK11) pada 2018/2020 manakala RM130,000.00 telah diperuntukan melalui Rancangan Malaysia Ke-12 (RMK12) pada 2021.

Jadual 3: Perincian Peruntukan dana untuk penyelidikan AHPND

Kajian	Perkara	Pecahan perbelanjaan
1.Status semasa kejadian AHPND di Malaysia	Analisis Bakteriologi	25,000.00
	Analisis Virus(kit pengesanan IMNV)	25,000.00
	Analisis Histopatologi	35,000.00
	Analisis Toksin PSP	12,000.00
2.Pencegahan dan kawalan ke atas AHPND di makmal dan ladang	Diagnosis kehadiran sel HP di Usus	10,000.00
	Diagnosis Bakteria	10,000.00
	Diagnosis patologi	10,000.00
	Eksperimen di makmal	13,000.00
	Eksperimen di kolam	10,000.00
	Hubungkait mutu air dengan kejadian AHPND (diagihkan ke FRI Pulau Sayak)	50,000.00
3. Pembangunan kawalan AHPND fasa I	Kajian petunjuk kesihatan udang ternak (Dana NKEA - ETP: 2014)	150,000.00
4. Pembangunan kawalan AHPND fasa II	Kajian kawalan bersama penternak (Dana NKEA - ETP: 2015)	200,000.00
5. Kajian pasca jangkitan AHPND di Malaysia	Pasca-AHPND di 9 negeri (Dana RMK11: 2018-2019)	150,000.00
6. Pembangunan rawatan AHPND	Saringan bahan mesra alam (Dana RMK11: 2015-2021)	80,000.00
7. Pembangunan kawalan dan rawatan AHPND	Percubaan di makmal dan lapangan (Dana RMK12: 2021)	50,000.00
Jumlah peruntukan yang diterima		**480,000.00**
Perbelanjaan sebenar		**479,046.35 (99.8%)**

4.2 Komponen penyelidikan

Lima komponen utama kajian berfokus bermula dari tahun aduan penternak mengenai kematian mendadak di peringkat awal ternakan pada 2011 sehingga 2021 (Rajah 5). Tinjauan data dari laporan kes diagnostik bermula dari tahun 1997 hingga 2020 juga turut diteliti bagi kajian survei kejadian AHPND.



Rajah 5: Komponen utama dalam kajian AHPND dari 1997 hingga 2021

4.3 Metodologi

4.3.1 Penentuan status kejadian AHPND di Malaysia

Kajian epidemiologi penyakit udang putih (*P. vannamei*) tidak pernah dilakukan sebelum 2011 di Malaysia. Program epidemiologi udang marin ternak hanya bermula selepas kejadian EMS/AHPNS dan melalui kerjasama antara NaFisH dan FRI Pulau Sayak, Institut Penyelidikan Perikanan ke atas ladang dari negeri Perak dan Kedah yang terjejas teruk akibat EMS/AHPNS. Program yang sama diteruskan di negeri Pulau Pinang, Pahang dan Sabah dan Sarawak pada tahun 2013. Halangan utama program pada masa tersebut ialah tiada peruntukan khas untuk penyelidikan penyakit baru. Justeru itu, kajian diteruskan dengan menggunakan semua kemudahan yang sedia ada dan disokong oleh pihak industri sendiri (penternak dan makmal swasta). Penentuan indikator kesihatan udang melalui ujian *hemolymph anti-clotting,* pemerhatian tanda klinikal dan persampelan dilakukan ke atas ladang yang terjejas. Manakala pengesanan penyakit EMS tertumpu kepada kaedah histologi dan interpretasi slaid histologi disahkan oleh Dr. Lighter dari Universiti Arizona, USA. Hubungkait kejadian penyakit turut berfokus kepada kajian mutu air dari kolam yang terjejas, kehadiran penyakit virus udang, penyakit bakteria udang disamping kajian toksin dari plankton dan tisu udang yang menunjukkan

tanda-tanda patologi dan kematian tinggi. Penyakit virus udang yang diuji terdiri daripada penyakit virus bintik putih (WSD/WSSV), *Infectious hypodermal and hematopoietic necrosis* (IHHNV), *Penaeus vannamei nodavirus* (PvNv), *Necrotizing hepatopancreatitis* (NHP) dan *infectious myonecrosis virus* (IMNV). Analisis penyakit virus udang menggunakan kit komersial manakala analisis toksin menggunakan kit ELISA kuantitatif PSP dan interpretasi keputusan disahkan oleh Dr. Yoshinobu Takata dari Kitasato Universiti, Jepun.

4.3.2 Survei kejadian AHPND dari pelaporan kes diagnostik

Laporan diagnostik bermula dari tahun 1997 turut dirujuk untuk melihat kepada pola kejadian serangan penyakit pada udang ternak marin untuk menentukan kejadian EMS/AHPND adalah penyakit baru semasa tahun kejadian. Bilangan kes setiap tahun dibanding dari segi punca penyakit, peringkat yang dijangkiti serta kerugian akibat penyakit tersebut. Perbandingan data dari laporan diagnostik dari tahun 1997 sehingga 2020 dilakukan melalui kompilasi kes diagnostik (Kua & Padilah, 2018, 2018b; Afzan Muntaziana et al., 2018; Mohd Syafiq et al., 2018 dan Rimatulhana et al., 2021).

4.3.3 Status pasca-AHPND selepas laporan pertama di Malaysia

Status prevalens AHPND ditentukan pada ternakan udang marin iaitu udang putih (*P. vannamei*) dan udang harimau (*P. monodon*) daripada peringkat pasca-larva (PL), juvenil atau Day of Culture (DOC) dan induk. Jangkitan AHPND dan prevalens bakteria *V. parahaemolyticus* daripada udang marin dihubungkait dengan kehadiran bakteria tersebut di dalam sistem kultur ternakan udang. Kajian 'cross-sectional' menggunakan persampelan secara kluster pada tiga peringkat kultur ternakan terdiri daripada PL, DOC dalam ternakan kolam dan induk dipilih secara rawak dalam satu kali persampelan bermula daripada November 2018 sehingga Oktober 2019 (Jadual 4). Pengesanan toksin gen *PirA/B* daripada tisu hepatopankreas udang dikenalpasti melalui kaedah polymerase chain reaction (PCR) berdasarkan kaedah rujukan manakala isolasi dan identifikasi bakteria dijalankan menggunakan kaedah kultur diikuti dengan ujian biokimia menggunakan Kit API bioMerieux (Analytical Profile Index) API 20NE atau API 20E.

Pengesahan kehadiran positif AHPND dijalankan menggunakan kaedah nested PCR dengan primer AP4 (Jadual 4 dan 5) (Dangtip et al., 2015) dan PreMix Maxime i-Taq (iNtRON Biotechnology, Korea). Pengesanan toksin gen *ToxA* dan *ToxB* atau *PirA/B* pada 230 bp iaitu merangkumi penjujukan gen 209 bp *ToxA* plus 12 bp 'spacer sequence' plus 9 bp penjujukan gen *ToxB* (Jadual 6).

Jadual 4. Jadual persampelan kajian *cross sectional* epidemiologi penyakit AHPND pada udang putih (*P. vannamei*) di Malaysia pada tahun 2019

Bil.	Negeri	Daerah	Tarikh persampelan	Jenis sampel	Bilangan sampel (n)
1	Kedah	Sungai Petani	Nov. 2018	Induk	50
		Alor Setar	Apr. 2019	DOC	30
2	P. Pinang	Balik Pulau	Feb. 2019	paska-larva	30
3	Perak	Manjung	Feb. 2019	DOC	30
4	Terengganu	Setiu	Mac 2019	DOC	30
5	Sarawak	Kuching Sarikei Miri	Feb. 2019 Mei, 2019 Mei, 2019 Mei, 2019	Induk DOC DOC DOC	21 30 30 30
6	Selangor	Kuala Selangor	Jun 2019	paska-larva	30
7	Johor	Batu Pahat Kota Tinggi	Ogos, 2019	DOC DOC	30 30
8	Pahang	Pekan Kuantan	Okt. 2019	DOC DOC	30 30
9	Sabah	Kudat Tawau	Nov. 2019	DOC DOC	30 30

Jadual 5. Primer-primer PCR berdasarkan kaedah rujukan Dangtip et al., 2015

Primer	5'-3'	Panjang	%GC	Tm	Ta	Jangkaan amplifikasi
AP4-F1	ATGAGTAACAATATAAAACATGAAAC	26	23	49	55	1269 bp
AP4-R1	ACGATTTCGACGTCCCAA	20	50	52		
AP4-F2	TTGAGAATACGGGACGTGGG	20	55	54	55	230 bp
AP4-R2	GTTAGTCATGTGAGCACCTTC	21	48	52		

Jadual 6: Kondisi tindakbalas 'nested' PCR

Komponen	Isipadu akhir	Protokol
Primer (F2: 100 pmol/µl) Primer (R2: 100 pmol/µl) Air suling ternyahion Templat DNA	0.4 µl 0.4 µl 17.2 µl 2 µl	Denaturasi 94 °C, 2 min 25 kitaran Denaturasi 94°C, 30 sec *Annealing* 55°C, 30 sec sambungan 72 °C, 90 sec Final 72 °C, 2 min
Jumlah isipadu	**20 µl**	

Jadual 7: Kondisi tindakbalas first PCR mengesan toksin gen *PirA/B* dan plasmid

Komponen	**Isipadu akhir**	**Protokol**
Primer (F1: 100 pmol/µl) Primer (R1: 100 pmol/µl) Air suling ternyahion Templat DNA	0.4 µl 0.4 µl 17.2 µl 2 µl	Denaturasi 94 °C, 2 min 30 kitaran Denaturasi 94°C, 30 sec *Annealing* 55°C, 30 sec Sambungan 72 °C, 90 sec Final 72 °C, 2 min
Jumlah isipadu	**20 µl**	

4.3.4 Pembangunan kaedah kawalan dan rawatan AHPND

Pengesahan kejadian AHPND pada tahun 2011 di Malaysia dan penemuan etiologi penyakit tersebut pada tahun 2013 seterusnya telah membawa kepada beberapa siri kajian berfokus kepada pencegahan atau rawatan di kebanyakan negara yang menternak udang marin. Di Malaysia, kajian mengenai kawalan samada di peringkat makmal dan ladang telah bermula sejak 2014 dan ia berterusan sehingga 2021. Langkah awal di peringkat makmal adalah menyaring bahan mesra alam yang mudah dan boleh diperolehi pada masa tersebut. Hasil saringan bahan dengan nilai aktiviti antimikrobial yang tinggi seterusnya diuji di makmal basah dan lapangan sebelum diperkenalkan kepada penternak udang marin.

Kesemua bahan mentah kajian dibeli daripada pembekal tempatan manakala sebahagian minyak pati komersial dibeli dari luar negara. Tiga jenis surfaktan ester lipid iaitu PT2, PT6 dan PT8 digunakan dalam ujian mengesan aktiviti antimikrob terhadap bakteria *V. parahaemolyticus*. Perbezaan antara surfaktan ester lipid yang dibekalkan ialah nisbah dan panjang rantaian lipid. Kepekatan yang digunakan untuk setiap ujian ditentukan pada 0.08% seperti yang disyorkan oleh pembekal. Minyak pati komersial

yang diperolehi terdiri dari ekstrak herba tumbuhan terdiri daripada kulit kayu manis (EOCIN), limau nipis, lemon, bawang putih dan serai wangi diuji untuk mengesan aktiviti antimikrobial terhadap 10 *V. parahaemolyticus* isolat yang diasingkan daripada udang berpenyakit AHPND.

i) Kaedah saringan aktiviti antimikrob bahan mesra alam sebagai kawalan

Beberapa ekstrak daripada tumbuhan dan mikroalgae diuji untuk mengesan aktiviti antimikrob terhadap beberapa bakteria patogen udang. Saringan bahan tersebut adalah terdiri daripada produk bahan buangan kelapa sawit yang dikenali sebagai i) surfaktan ester lipid dan bahan dari pengekstrakan tumbuhan seperti ii) buah, daun dan bunga kerukup siam; iii) biji buah Avocado (*Persia americana*); iv) daun hempedu bumi 'Bitter king' (*Andrographis paniculata*); v) Dukung anak (*Phyllanthus amarus*) vi) Eucabiotics (Eucalyptus oil); vii) Microalgae *Chlorella vulgaris*; viii) *Nannochloropsis* sp.; ix) *Isochrysis* sp.; x) *Rhodomonas* sp.; xi) sireh (*Piper betle*) dan xii) serai (*Cymbopogon citrates*). Selain daripada itu, penyaringan ke atas 5 jenis minyak pati komersial terdiri daripada i) kulit kayu manis, *Cinnamomum zeylanicum* (EOCIN), ii) limau nipis (*Citrus aurantiifolia*), iii) lemon (*Citrus limon*), iv) bawang putih (*Allium sativum*) dan v) serai wangi (*Cymbopogon nardus*) juga dikaji untuk melihat kesannya ke atas *V. parahaemolyticus* patogen.

Larutan lima minyak pati komersil disediakan dalam empat kepekatan iaitu 100%, 50%, 5% and 2.5% menggunakan etanol sebagai pelarut. Manakala bagi ekstrak 11 bahan tumbuhan, proses pengekstrakan untuk memperoleh minyak pati dilakukan melalui proses pembersihan, pemotongan, pengeringan dan dikisar menggunakan mesin pengisar makmal. Ekstrak yang diperoleh kemudiannya ditapis, disejat hingga kering dengan penyejat berputar dan diulang semula dalam etanol untuk mencapai kepekatan stok 100 mg/mL. Ekstrak seterusnya disimpan dalam botol gelap pada suhu 4^{o}C sehingga digunakan selanjutnya. Kesemua saringan bahan mesra alam ini seterusnya dilakukan aktiviti antimikrob dengan menggunakan bakteria *V. parahaemolyticus* yang diperolehi semasa kajian 2011 hingga 2012.

Pengesahan bakteria adalah mengikut kaedah DePaola et al. (2003) manakala kaedah aktiviti antimikrob surfaktan ester lipid mengikut Bauer et al. (1966) dan Anderson (1974). Aktiviti antimikrobial bagi 11 bahan dari tumbuhan dan minyak pati komersial adalah menggunakan kaedah cakera serapan agar (Disc diffusion agar) mengikut Bauer et al. (1966).

Penyediaan isolat bakteria untuk kaedah serapan cakera bermula dengan isolat *V. parahaemolyticus* dikultur dalam Trypticase Soy Agar (TSA) mengandungi 1.5 % NaCl. Kultur agar disimpan dalam inkubator pada 30^{o}C selama 18-24 jam. Selepas semalaman, 2 atau 3 koloni yang tumbuh atas TSA diambil untuk menyediakan larutan bakteria dalam saline (1 mL) dengan kepekatan sel sebanyak 10^8 CFU/mL (0.5 MacFarland Standard). Sebanyak 100 µL larutan bakteria disebarkan ke atas piring media MHA secara rata sebelum cakera ujian diletakkan ke atas Mueller Hinton Agar (MHA, Oxoid, UK) bersama antibiotik cakera dan cakera kawalan (pelarut /medium larutan bahan ujian).

Kaedah cakera serapan agar dijalankan menggunakan MHA di dalam piring petri bersaiz 90 mm dengan ketebalan agar pada 4 mm untuk memastikan serapan bahan aktif ujian berlaku pada kadar optimum. Sebanyak 100 µL bakteria sel (10^8 CFU/mL sel bakteria dalam 0.9% NaCl) dalam larutan saline disebarkan secara rata di atas piring petri MHA menggunakan L-shape steril plastik rod. Selepas 15 minit, cakera ujian kosong (6 mm diameter) (Oxoid, UK) direndam dengan bahan ujian iaitu EOCIN di dalam pelarut etanol pada kepekatan yang berbeza (100%, 50%, 5% dan 2.5%). Salah satu cakera direndam dengan 50% etanol (15 uL) sebagai kawalan. Plat agar ujian dimasukkan ke dalam oven inkubator pada suhu 30ºC selama 18-24 jam dalam keadaan aerobik. Setiap ujian dijalankan dengan tiga replikasi bagi setiap isolat bakteria. Selepas 24 jam, diameter zon rencatan cakera, kawasan di mana tidak terdapat pertumbuhan bakteria dari cakera mengandungi bahan ujikaji diukur dalam Diameter Zon Rencatan (DIZ) unit mikrometer (Jadual 8).

Jadual 8. Kategori Diameter Zon Rencatan (DIZ) bagi mengesan aktiviti antimikrob daripada tumbuhan herba dan minyak pati EOCIN (Zakaria et al. 2006)

Bil.	Paras Zon Rencatan	Diameter Zon Rencatan (DIZ)	Aktiviti antimikrob
1.	-ve	Tiada zon rencatan	Tiada aktiviti antimikrob
2.	+	DIZ ≤ 9 mm	Sangat rendah
3.	++	DIZ > 9 sehingga ≤ 12 mm	Rendah
4.	+++	DIZ > 13 sehingga ≤ 16 mm	Sederhana
5.	++++	DIZ > 16 ≤ 20 mm	Tinggi
6.	+++++	DIZ > 20 mm	Sangat tinggi

ii) Kaedah rawatan menggunakan surfaktan ester lipid di makmal dan lapangan

Surfaktan ester lipid yang mempunyai aktiviti antimikrob sederhana seterusnya diuji di peringkat makmal basah dengan menggunakan udang putih marin yang telah dijangkiti AHPND. Justeru itu, satu eksperimen di makmal basah dijalankan ke atas 36 ekor udang putih dari kolam yang telah mengalami 40-50% kematian udang akibat AHPND. Objektif kajian ialah untuk menilai kesan surfaktan ester lipid terhadap berat badan dan kadar kemandirian pada udang putih ternak yang telah dijangkiti AHPND. Udang hidup positif AHPND telah dipindah dari kolam ke makmal basah Institut Penyelidikan Perikanan dengan menggunakan bag plastik berisi air bersaliniti 23 ppt. Udang putih seterusnya dibahagi kepada dua kumpulan dan diletak di tangki berkapasiti 50 L yang dilengkapi dengan pengudaraan berterusan dan air masin bersaliniti 23 ppt. Setiap kumpulan mempunyai enam replikat dengan pengisian tiga ekor udang per tangki. Kumpulan

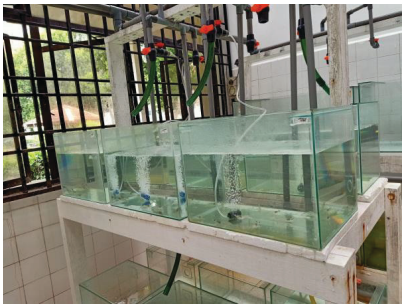
pertama diberi makanan pelet bercampur 0.08 ml/L surfaktan ester lipid selama 42 hari manakala kumpulan kedua menerima makanan pelet biasa tanpa campuran surfaktan ester lipid. Eksperimen yang sama diulang ke atas 48 ekor udang putih dari kolam yang mengalami serangan AHPND dengan tambahan 2 replikat. Kedua-dua eksperimen udang berpenyakit diperolehi dari ladang yang telah disahkan diserang AHPND dan menunjukkan tanda-tanda klinikal kematian awal kurang daripada 40 hari kultur (Jadual 9). Pemerhatian kematian harian ke atas tangki udang yang menerima makanan bercampur surfaktan ester lipid dan tangki kawalan direkodkan untuk setiap kumpulan dari kedua-dua eksperimen. Di akhir setiap eksperimen, semua udang hidup dari setiap replikat diambil untuk ukuran berat badan, catitan untuk kehadiran bakteria dan pengesahan AHPND melalui kaedah histologi (Bell & Lightner, 1988).

Jadual 9: Sejarah udang putih yang digunakan dalam dua eksperimen di makmal.

	Eksperimen Pertama	**Eksperimen Kedua**
Sumber udang yang dijangkiti AHPND	Kedah	Kedah
Kematian pertama yang direkodkan (tempoh ternakan dalam kolam (DOC)	22-23	22-23
DOC disahkan AHPND	25-28	25-28
Kehadiran jenis bakteria	*V. parahaemolyticus*	*V. alginolyticus*
Patologi AHPND (*Karyomegaly, sloughing of epithelial cell from hepatopancreas and melanized hepatopancreas tubules)*	positif AHPND	positif AHPND

iii) Kaedah makmal aplikasi minyak pati komersial kayu manis pada udang putih berusia 30 hari (DOC 30) sebagai kawalan AHPND di makmal

Satu regim pemberian makanan minyak pati komersial kayu manis (EOCIN) (1.5% isipadu/berat selama 2 minggu berturut-turut) diberi sebagai aditif di dalam pelet makanan dan ketahanan udang putih menghadapi cabaran bakteria *V. parahaemolyticus* diuji selepas tempoh 30 hari pertama ternakan. Kajian di lapangan pada ternakan tangki superintensif *P. vannamei* dijalankan di sebuah ladang swasta di Telok Tempoyak, Pulau Pinang pada Oktober, 2020. Selepas tamat dua minggu pemberian makanan bercampur EOCIN, udang tersebut di bawa ke makmal FRI/NaFisH untuk ujian cabaran jangkitan bakteria isolat *V. parahaemolyticus Vp14* AHPND. LD_{50} *V. parahaemolyticus* bakteria pada 3.16×10^7 cfu/mL kepekatan sel diuji secara suntikan intramuscular (IM) kepada udang putih berumur 30 hari (DOC 30) (Foto 1 - 4).



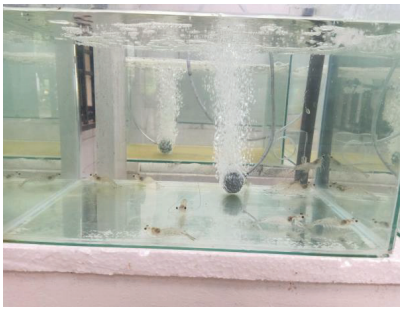
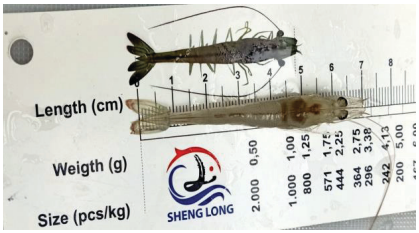


Foto 1 & 2: Tangki eksperimen mengandungi udang putih (10 ekor/tangki) dengan tiga replikasi bagi udang diberi makanan pelet bercampur dengan EOCIN dan kawalan



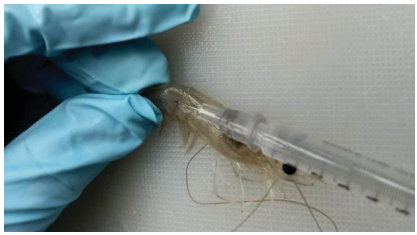


Foto 3 & 4: Udang putih *P. vannamei* DOC 30 dicabar dengan *V. parahaemolyticus* isolat Vp14 secara suntikan *intramuscular* (IM).

iv) Kaedah aplikasi minyak pati kayu manis komersial dalam diet makanan udang putih di lapangan

Kajian lanjut dijalankan untuk mengenalpasti potensi EOCIN dalam pencegahan penyakit AHPND pada ternakan udang putih. Kajian lapangan aplikasi EOCIN dijalankan dalam sistem tangki pada udang putih berkepadatan tinggi bersama syarikat tempatan di Teluk Tempoyak, Pulau Pinang (Foto 5 - 6). Kajian untuk tempoh satu pusingan bermula dengan penyaringan PL udang yang bebas dari penyakit. Hanya PL yang negatif dari AHPND digunakan dalam kajian seterusnya. Satu regim pemberian EOCIN pada dos 1.5% (v/w) dicampur dalam pelet makanan biasa dan diberi kepada udang selama 14 hari secara berterusan (DOC 1 - 14), diikuti dengan 7 hari tanpa EOCIN dan seterusnya udang diberi makan EOCIN 7 hari (DOC 22 - 28) (Foto 7). Udang seterusnya diberi makanan biasa selama seminggu (DOC 29 - 35) dan diikuti pulak dengan pemberian makanan bercampur EOCIN selama 14 (DOC 36 -49). Selepas itu, udang diberi makanan pelet tanpa campuran EOCIN sehingga udang mencapai saiz pasaran atau mencapai berat 100 ekor/kg. Pemantauan status kesihatan udang dipantau secara berkala melalui

aktiviti persampelan udang pada DOC 15 hingga DOC 50 untuk saringan mengesan penyakit AHPND dengan kaedah polymerase chain reaction (PCR). Pertumbuhan berat badan (g) dan panjang udang (cm) direkodkan bagi setiap aktiviti persampelan dijalankan. Kajian dijalankan dengan mengadakan tiga replikasi tangki ternak bagi udang menerima makanan bercampur EOCIN dan satu replikasi sebagai kawalan. Ternakan udang adalah secara superintensif pada kepadatan 350 ekor/m^3 dengan muatan sebanyak 300,000 ekor udang bagi setiap tangki.

Foto 5: Tangki berkapasiti tinggi yang digunakan dalam kajian lapangan.

Foto 6. Kajian EOCIN di kolam ternakan udang putih sistem tangki superintensif

Foto 7. Pemerhatian udang dalam dulang makanan

v) Kaedah pengesanan AHPND dengan skor kad usus udang di makmal

Semasa kajian awal siasatan kemunculan penyakit AHPND pada 2011 hingga 2013, maklumat penting yang ditemui ialah udang yang dijangkiti oleh AHPND boleh menyingkirkan sel epitelium di tubul hepatopankreas yang seterusnya disalur ke usus udang untuk tujuan pembuangan oleh udang. Udang yang tidak sihat atau berpenyakit biasanya menunjukkan tanda-tanda klinikal dan pada masa yang sama menunjukan proses pemulihan. Pada tahap ini, tindakan segera perlu diambil bagi mengelakkan kematian. Penemuan kehadiran sel hepatoprankreas dalam usus udang sakit boleh digunakan sebagai petunjuk kepada udang yang dijangkiti oleh AHPND.

Pengesahan hubungkait antara maklumat ini dengan petunjuk kehadiran sel epitelium hepatopankreas di usus telah diuji di peringkat makmal. Sejumlah 150-300 udang berusia 22 hari ternakan telah diperolehi dari beberapa kolam ternakan di Perak. Hanya kumpulan udang yang bebas dari penyakit AHPND digunakan untuk ujian cabaran dengan patogen bakteria pencetus AHPND. Kumpulan udang putih bebas AHPND seterusnya dicabar dengan patogen bakteria *V. parahaemolyticus* dan pemerhatian kehadiran sel epitelium tersingkir direkodkan dalam tempoh 24 jam di makmal basah. Pasca larva udang yang dijangkiti oleh AHPND turut disahkan dengan kehadiran sel epitelium tersingkir di organ hepatopankreas melalui kaedah histologi. Tahap jangkitan AHPND samada di awal atau akhir jangkitan turut dibuat dengan mengambil kira jumlah atau densiti kehadiran sel epitelium tersingkir berada di sepanjang usus udang. Densiti sel ini seterusnya dinilai dengan menggunakan skor bermula dari 0 hingga 4. Skor 0 merujuk kepada tiada penemuan sel epitelium di usus, skor 1 dan 2 adalah penemuan jumlah sel epitelium berada di bawah separuh daripada panjang usus manakala skor 3 & 4 merujuk kepada kehadiran sel epitelium melebihi daripada separuh panjang usus udang.

vi) Kaedah pengesanan AHPND dengan skor kad usus udang putih di kolam berskala besar

Pengesahan hubungkait kejadian AHPND dengan kehadiran sel epitelium tersingkir ini juga disahkan di peringkat lapangan dengan mengenalpasti kolam udang yang belum dan telah dijangkiti AHPND. Sampel udang yang belum dan telah dijangkiti disahkan dengan kadar skor dan seterusnya ditentusahkan dengan kaedah histologi dan PCR. Kajian telah dijalankan di sebuah ladang ternakan udang putih di Perak yang melibatkan 2 pusingan ternakan udang putih. Bagi ternakan pusingan pertama, penyampelan dilakukan pada DOC 30, 52, 72 dan 87 manakala bagi ternakan pusingan kedua, DOC 15, 30, 50, 65 dan 78 telah disampel. Analisis pengenalpastian bakteria dijalankan menggunakan kit API® bioMérieux Perancis manakala analisis PCR untuk penentuan jangkitan AHPND menggunakan kit IQ2000 ems2 (GeneReach, Taiwan). Bacaan skor usus diambil melalui pemerhatian usus udang dibawah mikroskop cahaya dan disahkan penyakit AHPND melalui analisis histopatologi.

5.0

KEPUTUSAN

5.1 Status pengesahan kejadian AHPND di Malaysia

Lapan belas penternak terjejas dari enam negeri (Perak, Kedah, Sarawak, Pahang, Pulau Pinang & Sabah) telah dipantau pada Okt 2011 hingga Jun 2012. Purata kadar kematian dari kolam terjejas adalah antara 6 hingga 97% (Jadual 10). Daripada 428 udang yang dikaji, 90-100% menunjukkan tompokan putih di bahagian badan perut, hepatopankreas bengkak atau gelap, kurang selera makan, pertumbuhan terbantut, kematian perlahan, najis putih dan badan lembut (Foto 8 - 12). Purata 80% udang menunjukkan masa pembekuan darah melebihi 1.5 minit, petunjuk kepada keadaan udang yang tidak sihat (Jadual 11). Ujian IMNV, PvNv dan NHP yang dijalankan menunjukkan keputusan negatif terhadap sampel yang diambil. Walau bagaimanapun, terdapat kes positif IHHNV di beberapa ladang (Jadual 12).

Sampel udang terjejas menunjukkan patologi AHPND seperti ketiadaan sel B, F atau R dalam organ hepatopankreas, nekrosis dan penyingkiran sel hepatopankreatik serta tubul nekrotik berkapsul haemosit (Foto 13). Selain itu, patologi septik multifokal, tubul hepatopankreatik termelanisasi dengan hemosit terkapsul juga diperhatikan (Foto 14 - 15). Kesemua perubahan histopatologi yang dilihat menunjukkan situasi akut AHPND. Perubahan patologi tersebut turut dikesan pada sampel udang putih bermula dari usia 10 hingga 60 hari ternakan.

Parameter kualiti air menunjukkan paras ammonia, nitrit, sulfida dan ferum yang tinggi. Kehadiran intensiti tinggi beberapa spesies diatom dan dinoflagellata dalam kolam kultur yang terjejas menunjukkan wujud risiko lain yang boleh menjadi faktor awal pencetus kejadian kematian udang ternak. Pengesanan toksin *Paralytic Shellfish Poison* (PSP) dengan teknik ELISA didapati positif pada sampel yang mengalami AHPND. Kehadiran toksin PSP dikesan dengan kadar tinggi di organ hepatopankreas (Rajah 6). Walaubagaimanapun kesan PSP toksin ini tidak memudaratkan manusia.

Jadual 10: Maklumat siasatan di enam negeri yang terjejas semasa tahun kejadian

Lokasi	Kedah	Sabah	P.Pinang	Pahang	Sarawak	Perak
Bilangan kolam terjejas	54	38	3	11	5	6
Sumber benih	SPF	SPF	SPF	SPF	SPF & bukan SPF	SPF & bukan SPF
Kadar pelepasan (m^2)	70	60 - 100	100	100 - 120	80 - 120	80 - 120
Sistem ternakan	Kolam	Kolam	Kolam	Kolam	Kolam	Kolam
pH **Suhu (°C)** **Tahap kemasinan (ppt)**	7.8 – 8.2 28 – 29 25	7.2 -8.3 - 25 – 33	- - 25 – 28	- - 28 – 30	- - 25 – 30	- - 25 – 28
Rawatan air masuk	Rawatan Chlorine (40ppm)	Tiada Rawatan	Rawatan Chlorine (40ppm)	Rawatan Chlorine (40ppm)	Rawatan Chlorine (40ppm)	Rawatan Chlorine (40ppm)
Tarikh kematian dikesan	Jun 2012	Jun 2011 – 2012	2011 – 2012	2011 – 2012	2011 – 2012	Jun 2010 – 2012
Saiz/berat udang	2 g	5 – 14 g	> 14g	>14 g	2 - 15g	2 - 10g
Anggaran Kematian	30 – 60%	20 – 30%	60%	6 – 76%	60 – 80%	73 – 97%
Tanda-tanda penyakit:						
a) Tidak makan		Ya	Ya		Ya	
b) Berenang tepi kolam		Ya				
c) Tumbesaran lambat		Ya	Ya		Ya	
d) Najis berwarna putih		Ya	Ya	Ya	Ya	Ya
e) Bintik hitam (badan)			Ya			
f) Insang berwarna hitam	Ya				Ya	
g) Badan lembut		Ya	Ya	Ya	Ya	Ya
h) Badan / ekor putih	Ya			Ya	Ya	Ya
i) Badan merah	Ya		Ya	Ya	Ya	Ya
j) Kepala kuning		Ya				
k)Hepatopankreas pucat		Ya	Ya	Ya	Ya	Ya
i) Lain-lain tanda	Kepala hitam, putih	*Soft shell*				

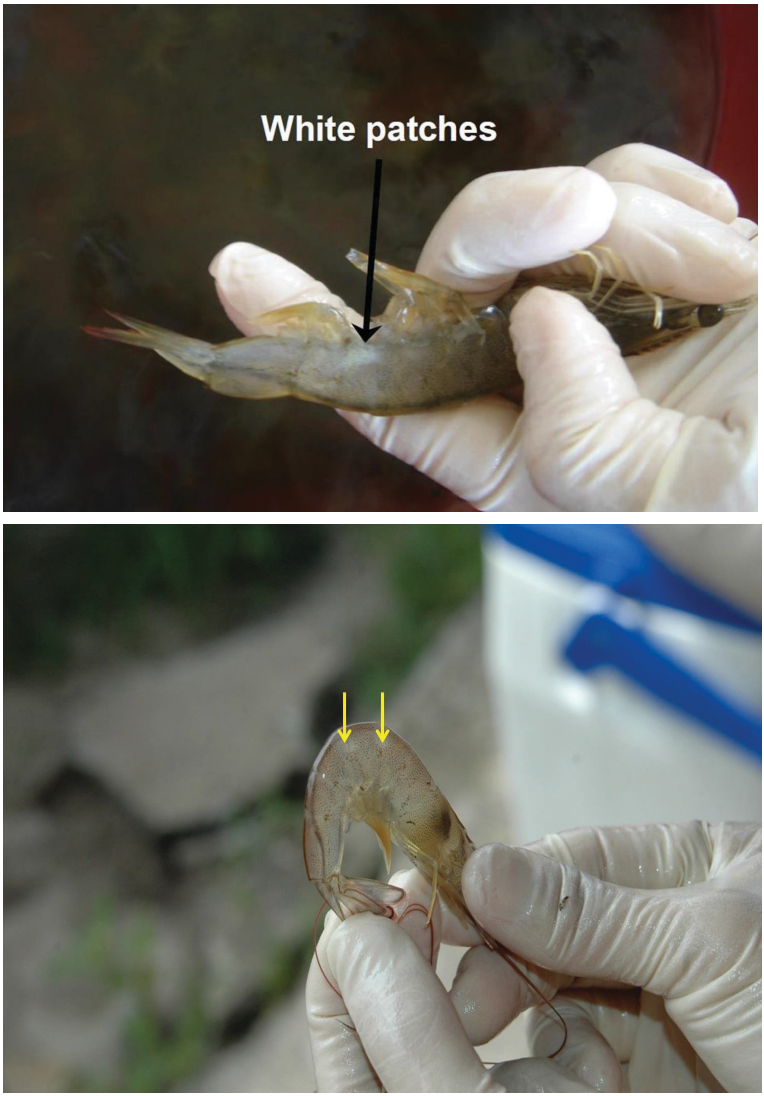


Foto 8: Tanda tompok putih (anak panah) di bahagian otot udang putih ternak

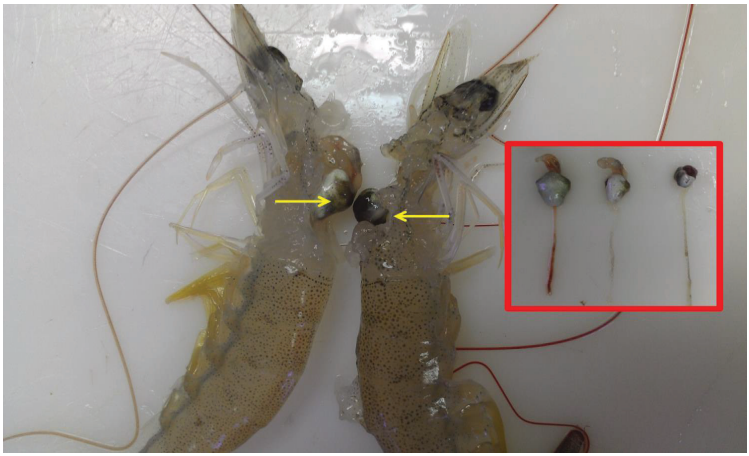


Foto 9: Saiz organ hepatoprankreas yang kecil (anak panah).

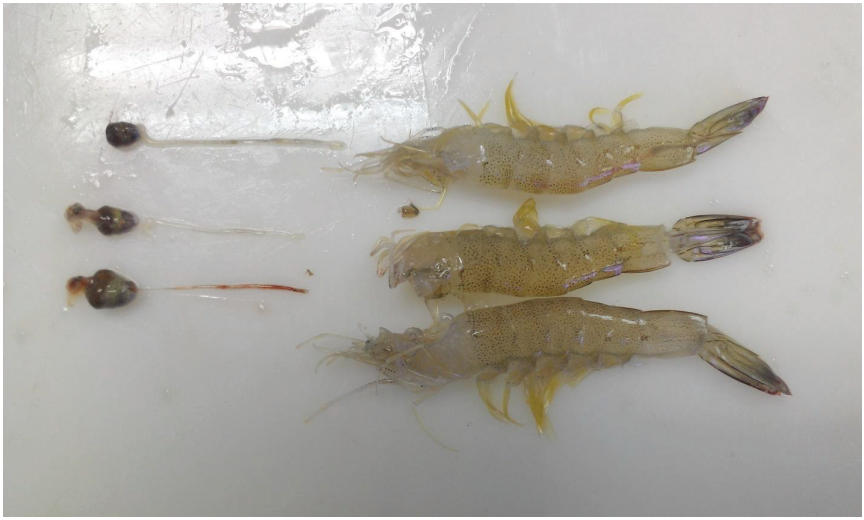


Foto 10: Perbezaan saiz organ hepatopankreas udang putih yang sama usia ternakan.

Foto 11: Udang putih yang mati di tepi kolam.

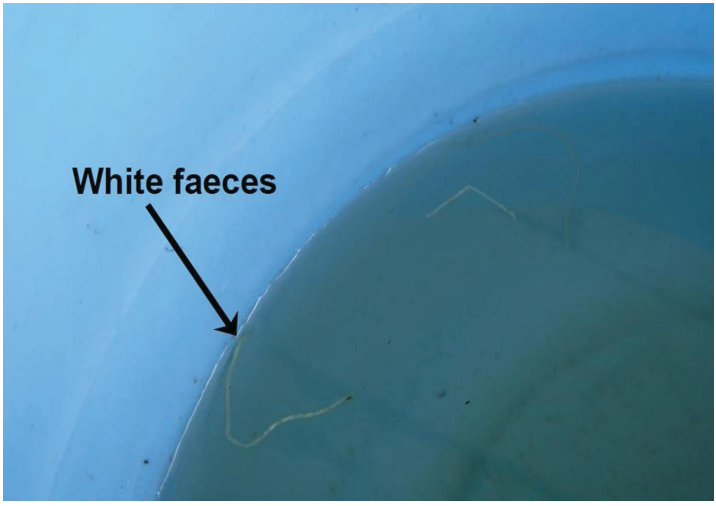


Foto 12: Najis putih yang ditemui semasa siasatan di tapak kejadian.

Jadual 11: Indikator kesihatan udang melalui ujian *hemolymph anti-clotting time*

Negeri	Bil. udang diuji	Bil. udang menunjukkan masa beku melebihi 1.5 minit	Bil. udang tidak sihat (%)
Perak	20	17	85
Pahang	30	15	50
Pulau Pinang	22	14	63
Kedah	10	10	100
Sabah	27	27	100

Jadual 12: Keputusan makmal bakteria, virus dan histologi bagi sampel yang diuji

Negeri	Penyakit Bakteria	Penyakit Virus	Patologi
Perak	*Vibrio* spp. *Photobacterium damselae*	7/64 +ve IHHNV	Peringkat awal dan akhir AHPND
Pahang	*Vibrio* spp.	0/110 –ve IHHNV 0/*20 –ve IMNV (Real-time* PCR)	Peringkat awal dan akhir AHPND
Pulau Pinang	*Tiada*	0/22 –ve IHHNV	Peringkat awal dan akhir AHPND
Kedah	*Vibrio* spp.	-	Peringkat akhir AHPND
Sabah	*Vibrio* spp.	0/41 –ve IMNV, PvNv & NHBP (IQ Plus) 0/3 –ve TSV(IQREAL) 11/41 +ve IHHNV	Peringkat awal dan akhir AHPND

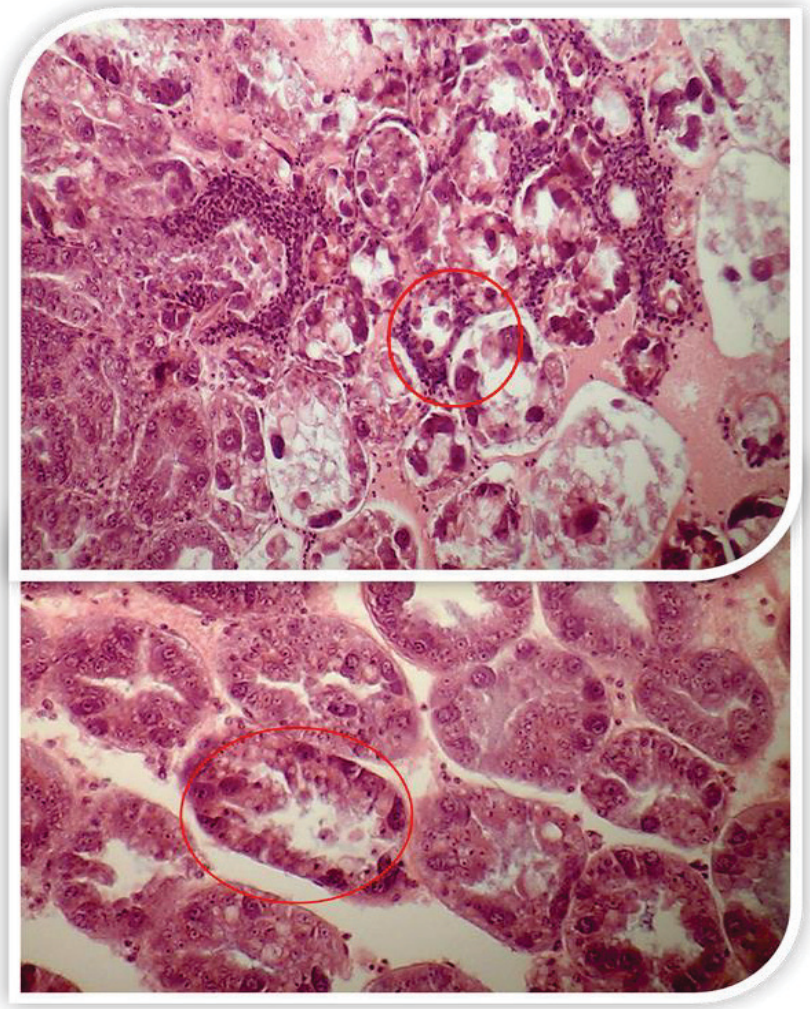


Foto 13: Patologi penyingkiran sel epitelium hepatopankreas ke dalam lumen tubul organ (bulatan merah)

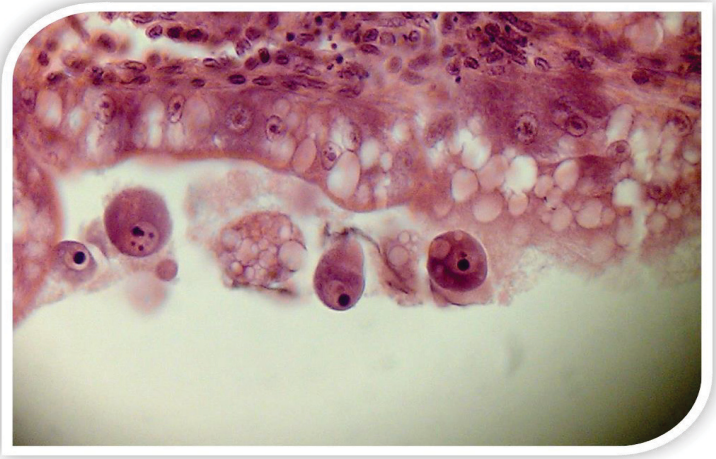


Foto 14: Patologi penyingkiran sel epitelium hepatopankreas ke dalam lumen tubul (kuasa pembesaran 60x).

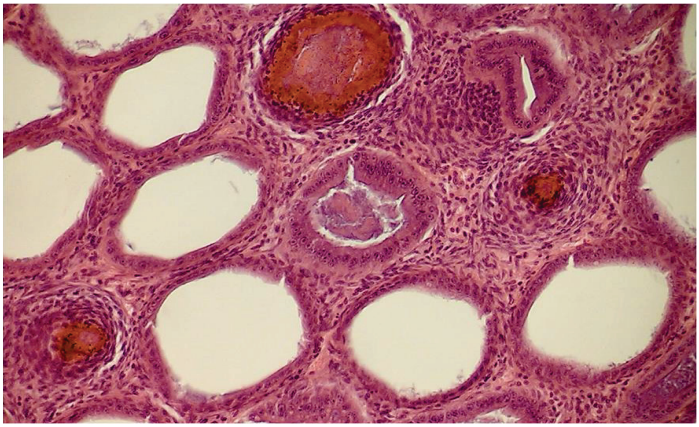
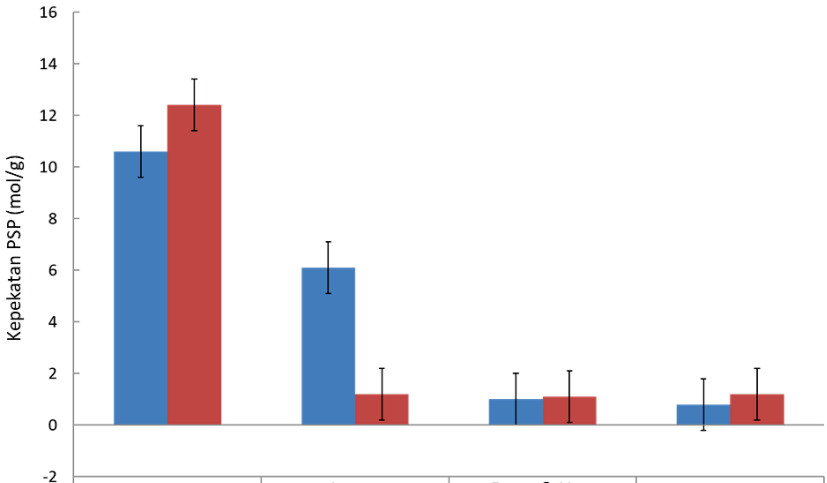


Foto 15: Patologi septik multifokal, tubul hepatopankreatik *termelanisasi* dengan hemosit terkapsul



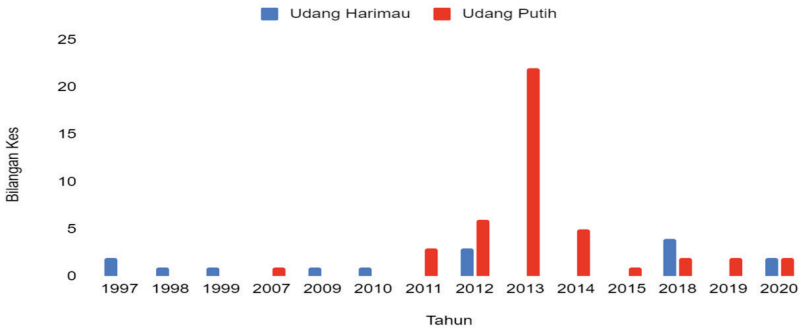
	Hepatopankreas	Insang	Perut & Usus	Otot
Nenasi	10.6	6.1	1	0.8
Pekan	12.4	1.2	1.1	1.2

Rajah 6: Kehadiran toksin PSP yang dikesan pada sampel udang yang dikaji

5.2 AHPND dalam kes pelaporan diagnostik udang ternak marin di NaFisH

Bilangan kes diagnostik pelaporan yang melibatkan spesis udang harimau dan udang putih sejak 1997 hingga 2010 ke NaFisH adalah dalam julat kurang dari 5 pelaporan setiap tahun (Rajah 7). Namun, sejak 2011, terdapat peningkatan kes diagnostik terutamanya ternakan spesis udang putih dengan bilangan kes masing-masing 4, 6, 22 pada 2011, 2012 dan 2013. Walau bagaimanapun, pelaporan kes mula menurun pada 2015 iaitu hanya 2 kes dan 3 kes setiap tahun bermula dari 2018, 2019 dan 2020. Peningkatan bilangan kes pelaporan mungkin disebabkan oleh program kesedaran mengenai penyakit baru pada udang putih yang menyebabkan ramai penternak menghantar sampel kepada NaFisH.

Anggaran kerugian di peringkat hatceri untuk pasca-larva bagi ternakan udang putih hanya direkodkan pada tahun 2012 dengan nilai kerugian berjumlah RM6,000 manakala RM3,041,775 bagi ternakan di tangki atau kolam yang bermula dari tempoh satu hari sampai saiz pasaran sepanjang tahun 2011 hingga 2022 (Jadual 13).



Rajah 7: Laporan bilangan kes diagnostik yang melibatkan udang ternak marin yang diterima oleh NaFisH sejak 1997 hingga 2020.

Jadual 13: Anggaran kerugian akibat penyakit AHPND pada ternakan udang di hatceri dan di tangki/kolam yang dilaporkan sepanjang 1997 hingga 2020.

a Laporan kerugian di hatceri

Tahun	Spesis	Punca	Peringkat mengalami kematian (Post-larva)	Jumlah post-larva/tangki	Bilangan tangki ternakan terjejas (Kapasiti 5 tan)	Jumlah post-larva dimusnahkan	Nilai Borong Post-larva (RM/post-larva)	Anggaran Jumlah Kerugian (RM)
2012	Udang Harimau	AHPND	8	100,000	3	300,000	0.02	6,000
							Jumlah	**6,000**

b Laporan kerugian di tangki/kolam ternakan (Tempoh ternakan satu hari hingga saiz pasaran)

Tahun	Spesis	Punca	Peringkat mengalami kematian (DOC=Tempoh Hari Ternakan)	Peratusan Kematian (%)	Jumlah Kolam Terjejas	Nilai Borong (RM/Tan)	Anggaran Jumlah Kerugian (RM)
2011	Udang Putih	AHPND	<DOC 30	50	2	12,194	12,194
	Udang Putih	AHPND	<DOC 30	100	1	12,194	12,194
	Udang Putih	AHPND	<DOC 30	50	1	12,194	6,097
2012	Udang Putih	AHPND	DOC 20 & 40	50	2	12,572	12,572
	Udang Putih	AHPND	DOC 20	100	11	12,572	138,287
	Udang Putih	AHPND	DOC 20	100	2	12,572	25,143
	Udang Putih	AHPND	<DOC 30	100	5	12,572	62,858
	Udang Putih	AHPND & WSSV	DOC 20	100	1	12,572	12,572
	Udang Putih	AHPND	DOC 20 & 40	50	2	12,572	12,572
2013	Udang Putih	AHPND	<DOC 30	100	5	15,924	79,620
	Udang Putih	AHPND	<DOC 30	100	5	15,924	79,620
	Udang Putih	AHPND	<DOC 30	100	3	15,924	47,772
	Udang Putih	AHPND	<DOC 30	100	4	15,924	63,696
	Udang Putih	AHPND	<DOC 30	100	2	15,924	31,848
	Udang Putih	AHPND	DOC 28	100	1	15,924	15,924
	Udang Putih	AHPND	<DOC 30	100	5	15,924	79,620
	Udang Putih	AHPND	<DOC 30	100	2	15,924	31,848
	Udang Putih	AHPND	<DOC 30	100	1	15,924	15,924
	Udang Putih	AHPND	<DOC 30	100	1	15,924	15,924
	Udang Putih	AHPND	<DOC 30	100	2	15,924	31,848
	Udang Putih	AHPND	<DOC 30	100	2	15,924	31,848
	Udang Putih	AHPND	<DOC 30	Tiada maklumat	3	-	-
	Udang Putih	AHPND	<DOC 30	100	1	15,924	15,924
	Udang Putih	AHPND	<DOC 30	100	13	15,924	207,013
	Udang Putih	AHPND	<DOC 30	100	8	15,924	127,393
	Udang Putih	AHPND	<DOC 30	100	19	15,924	302,557
	Udang Putih	AHPND	<DOC 30	100	3	15,924	47,772
	Udang Putih	AHPND	<DOC 30	100	1	15,924	15,924
	Udang Putih	AHPND	<DOC 30	100	1	15,924	15,924
	Udang Putih	AHPND	<DOC 30	100	8	15,924	127,393
2014	Udang Putih	AHPND	<DOC 30	100	12	19,871	238,454
	Udang Putih	AHPND	DOC 21	100	8	19,871	158,969
	Udang Putih	AHPND	DOC 22, 42 & 52	100	20	19,871	397,423
	Udang Putih	AHPND	<DOC 30	100	20	19,871	397,423
2015	Udang Putih	AHPND	DOC 27	50	12	21,092	126,551
2019	Udang Putih	EHP & AHPND	DOC 100 - 120	50	4	22,037	44,074
2020	Udang Putih	EHP & AHPND	>DOC 30	Tiada maklumat	8	-	-
						Jumlah	**3,042,775**

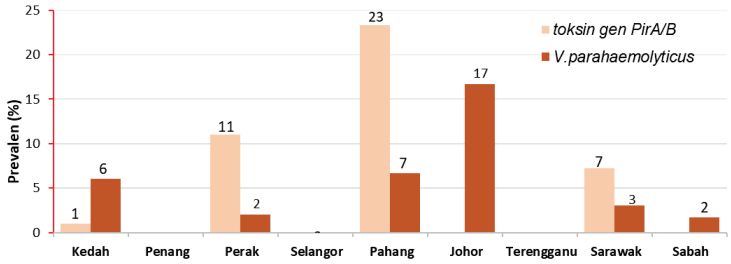
5.3 Status pasca-AHPND di sembilan negeri pengeluar udang ternak marin

Selepas tujuh tahun pengesahan AHPND yang pertama di Malaysia, jangkitan AHPND masih dikesan pada kedua-dua spesis udang ternak marin di sembilan negeri pengeluar utama dalam lingkungan julat 1 hingga 23%. Bagi spesis udang putih, prevalen 23% di kesan di Pahang, 11% di Perak, 7% di Sarawak dan hanya 1% dikesan di Kedah. Jangkitan AHPND juga dikesan pada udang harimau di Pahang, 10% dan Sarawak, 5% (Rajah 8). Jangkitan AHPND tidak dikesan pada udang putih di Pulau Pinang, Selangor, Johor dan Terengganu semasa tahun kajian.

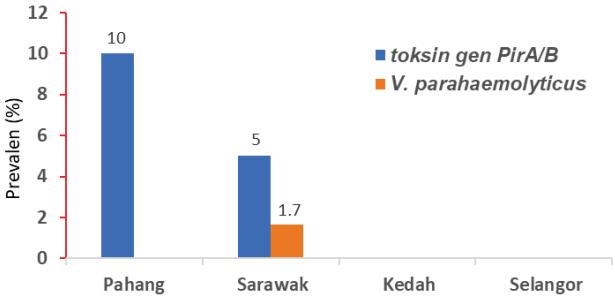
Kajian ke atas kadar jangkitan AHPND pada udang harimau dijalankan di beberapa negeri seperti Pahang, Sarawak, Kedah dan Selangor meliputi pusat pembenihan, hatceri dan asuhan benih di Kedah dan Pahang, pusat pembenihan (induk) dan ternakan udang di kolam di kedah, Selangor dan Sarawak. Secara am, AHPND adalah lebih rendah pada udang harimau (10% dan 6.7%) berbanding udang putih (23% dan 10%) di negeri Pahang dan Sarawak di mana kajian melibatkan kedua-dua spesies udang putih dan udang harimau (Rajah 9).

Kehadiran bakteria *V. parahaemolyticus* mempunyai toksin gen *PirA/B* yang menyumbang kepada kejadian AHPND dikesan pada hampir semua sampel kajian (Rajah 10). Prevalen *V. parahaemolyticus* bakteria adalah tinggi pada udang putih dari Johor (17%), Sabah (11%) dan Kedah (6%) dengan kehadiran toksin gen *PirA/B*. *Vibrio. vulnificus* mendominasi mikrobial di dalam organ hepatopankreas udang putih dengan prevalen tinggi di antara 20 - 38%.

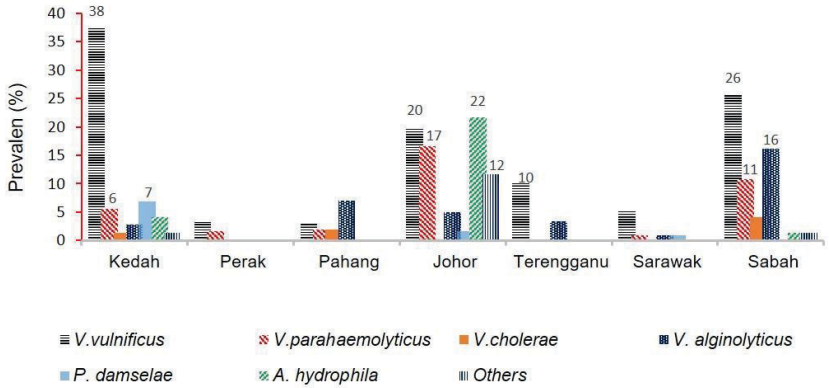
Penjujukan genomik dan filogenetik analisis lima isolat bakteria *V. parahaemolyticus* daripada udang putih yang dikesan positif AHPND pada tahun 2019 ditentukan menggunakan kaedah *next generation sequencing* (NGS). Perbandingan dibuat di antara penjujukan genomik (*assembled genomes*) strain tempatan dengan *Vibrio* spp. daripada Gene Bank (NCBI) untuk menentukan status isolat berkaitan di dalam pentaksiran taksonomi (*taxonomic assignment*). Fragmen gDNA ekstrak pada saiz 350 bp dikonstruk menggunakan NEB Ultra II DNA library sequenced pada Illumina NovaSeq 6000. Empat jujukan Genom daripada strain tempatan daripada Johor, Kedah dan Sarawak dengan identifikasi AAT22, IKK3, PK3 dan Vp14 menunjukkan purata identity 'pairwise' nukleotida melebihi 98% kepada *V. parahaemolyticus* manakala strain SK6 (Sabah) menunjukkan ANI sebanyak 98.54% to *V. alginolyticus*. *Alignment* dari penjujukan genomik keseluruhan empat *V. parahaemolyticus* isolat kepada pVA1 plasmid menunjukkan keputusan yang konsisten dengan ujian awal PCR mengesan toksin gen *PirA/B*. Hanya strain Vp14 menunjukkan homologi penjujukan penuh pVA1 plasmid manakala strain AAT22 (Johor) menunjukkan 50% penjujukan homologi plasmid mengandungi gen utama berkaitan fungsi pemindahan konjugatif tetapi tiada segmen *PirA/B* gen (Rajah 11 & 12).



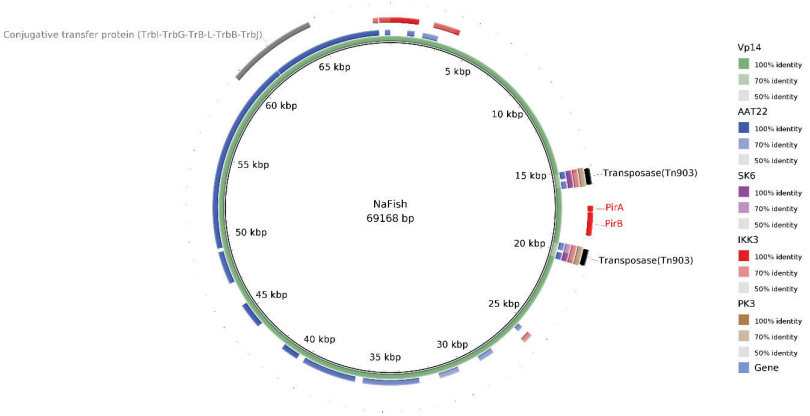
Rajah 8. Pengesanan toksin gen *PirA/B* dan *plasmid* daripada hepatopankreas udang positif AHPND dan kehadiran bakteria *V. parahaemolyticus* di dalam organ hepatopankreas membawa toksin gen *PirA/B* dan plasmid.



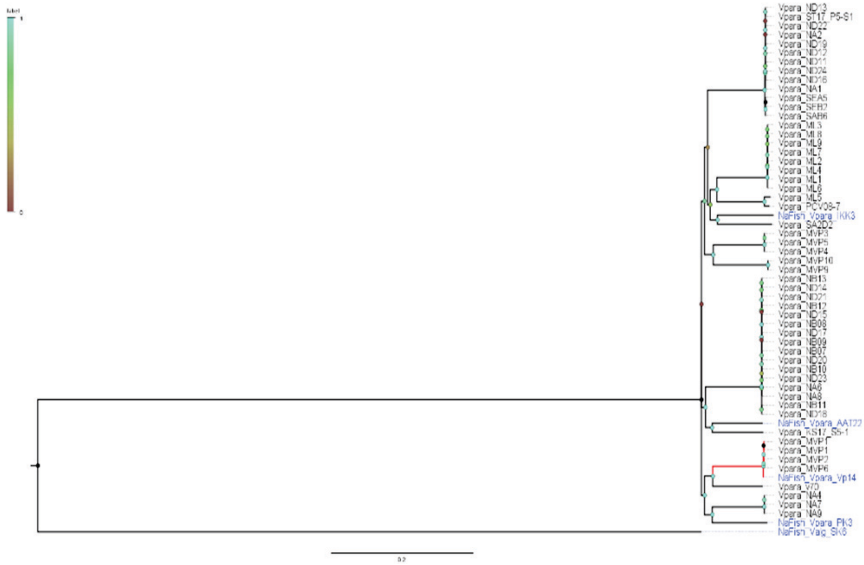
Rajah 9. Prevalen AHPND dengan pengesanan toksin gen *PirA/B* atau *ToxA*&*ToxB* dan pengasingan bakteria *V. parahaemolyticus* dengan pengesanan toksin gen *PirA/B* daripada organ hepatopankreas udang harimau.



Rajah 10: Peratus prevalen *V. parahaemolyticus* dikaitkan dengan jangkitan AHPND dan bakteria lain pada udang putih daripada kajian pada tahun 2019 di tujuh negeri Malaysia.



Rajah 11: Penjujukan penuh pVA1 plasmid bagi isolat tempatan Vp14 manakala isolat lain (AAT22, IKK3 dan PK3) hanya mengandungi plasmid tanpa toksin gen *PirA/B*



Rajah 12: *Maximum likelihood tree* dirangka berdasarkan kepada *SNP alignment* daripada *V.parahaemolyticus* genom terdapat di Malaysia dan NaFisH *Vibrio* genom (label biru). Strain SK6 adalah dasar (rooted) bagi kumpulan luar daripada pengkelasan taksonomi *V. alginolyticus*.

5.4 Saringan aktiviti antimikrob bahan mesra alam sebagai kawalan

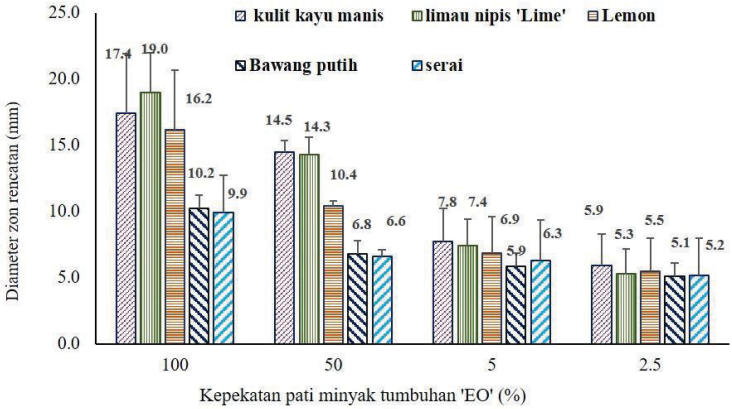
Kesemua minyak pati komersial yang diuji menunjukkan diameter zon rencatan terhadap *V. parahaemolyticus* AHPND patogen (Rajah 13, Foto 16 - 19). Minyak pati komersil limau nipis, kayu manis (EOCIN) dan lemon menunjukkan aktiviti antimikrob yang tinggi terhadap bakteria *V. parahaemolyticus* pada kepekatan asal (100%) dengan DIZ melebihi 16 mm manakala pada kepekatan 50%, antimikrob aktiviti berada pada paras sederhana (DIZ ≥ 12 - 16). Limau nipis menunjukkan aktiviti antimikrob paling tinggi pada kepekatan asal (100%) dengan DIZ daripada 11 - 26 mm (min, 19.00 ± 2.70), EOCIN, 8 - 23 mm (17.40 ± 3.90), lemon, 7 - 23 mm (16.20 ± 3.90), bawang putih, 4 - 14 mm (10.20 ± 2.10) dan serai wangi, 4 - 14 mm (9.90 ± 2.90).

Analisa ANOVA One-way menunjukkan aktiviti antimikrob daripada minyak pati komersil yang signifikan pada kepekatan 100% ($F_{4,44}$ = 12.898, P = 0.000; P ≤ 0.05) dan 50% ($F_{4,44}$ = 17.513, P = 0.000; P ≤ 0.05). Pati minyak limau nipis pada kepekatan asal (100%) menunjukkan aktiviti antimikrob yang tinggi pada 90% isolat *V. parahaemolyticus*

yang diuji (DIZ: 16 - 26 mm) diikuti dengan EOCIN (DIZ: 16 - 23 mm) daripada 70% isolat dan 60% minyak pati komersil lemon (DIZ: 16 - 23 mm). Antimikrob aktiviti minyak pati komersil bawang putih dan serai wangi adalah pada paras sederhana (DIZ: 12 - 16 mm) terhadap 30% dan 20% of isolat *V. parahaemolyticus*. Keputusan yang signifikan ($P \leq 0.05$) juga dikesan pada pati minyak komersil bawang putih dan serai wangi bagi aktiviti antimikrob (DIZ) yang sederhana terhadap *V. parahaemolyticus.*

Ekstrak sireh (SirehMax) *Piper betle* menunjukkan aktiviti antimikrob yang tinggi (DIZ>16mm) terhadap tiga isolat *V. parahaemolyticus* AHPND patogen (Vp14, EUS27 dan EUS12) yang diasingkan daripada udang putih dengan purata DIZ: 24.00 ± 1.00 mm, 21.30 ± 1.15 mm dan 21.30 ± 0.58 mm (Foto 20). Manakala aktiviti antimikrob yang rendah (DIZ ≤ 9 mm) dikesan bagi ekstrak biji avocado dan ceri hutan (Foto 21 - 22).

Ekstrak mikroalga (*C. vulgaris*, *Nanochloropsis* sp., *Rhodomonas* sp., *Isochrysis* sp.) dan serbuk Eucabiotics (minyak Eucalyptus >5%) menunjukkan aktiviti antimikrob yang rendah terhadap kebanyakan isolat patogen *V. parahaemolyticus* (Foto 23), antimikrob aktiviti dengan DIZ yang rendah juga dikesan daripada ekstrak *Nannochloropsis* sp. (≤ 9 mm). Zon rencatan pertumbuhan mikrob daripada ujian asai cerapan cakera terhadap *V. parahaemolyticus* tidak dikesan daripada ekstrak *C. vulgaris*, *Isochrysis* sp., *Rhodomonas* sp. serta ekstrak tumbuhan daun hempedu bumi dan dukung anak (Foto 24 - 27). Namun, pengekstrakan menggunakan pelarut metanol ke atas *C. vulgaris* menunjukkan zon rencatan yang rendah terhadap bakteria *V. parahaemolyticus* (DIZ: 1 - 4 mm) dan *V. alginolyticus* (DIZ: 4 – 5 mm). Jadual 14 menunjukkan ringkasan penemuan aktiviti antimikrob daripada ekstrak tumbuhan dalam kajian yang dijalankan dari tahun 2017 sehingga 2021.



Rajah 13: Diameter Zon Rencatan daripada lima minyak pati komersial terhadap isolat *V. parahaemolyticus* AHPND patogen.

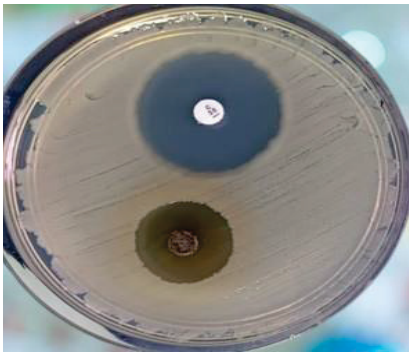


Foto 16. Aktiviti antimikrob yang tinggi (DIZ>16mm) daripada SirehMax terhadap *V. parahaemolyticus* (Vp14) dan sensitiviti isolat terhadap antibiotik Chloramphenicol (30ug/disc)

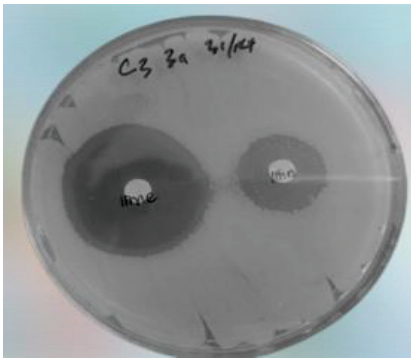


Foto 17: Aktiviti antimikrob yang tinggi (DIZ>16) daripada minyak pati komersil limau nipis dan lemon (kepekatan 100%) terhadap isolat *V. parahaemolyticus* AHPND patogen (C33a)

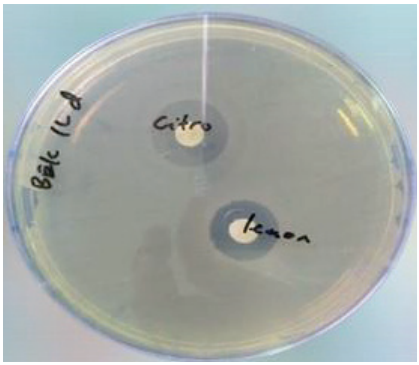


Foto 18: Aktiviti antimikrob yang sederhana (DIZ >12-16 mm) daripada minyak pati komersial serai wangi (citronella, 2.5%) dan lemon (2.5%) terhadap isolat *V. parahaemolyticus* AHPND patogen (BEK1Ld)

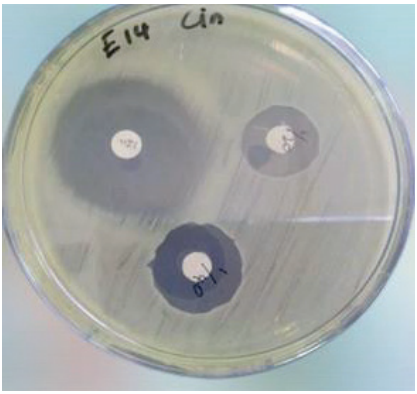


Foto 19: Aktiviti antimikrob yang sederhana bagi minyak pati kayu manis komersial (DIZ: >12 sehingga ≤16 mm) pada kepekatan 5% dan 2.5%

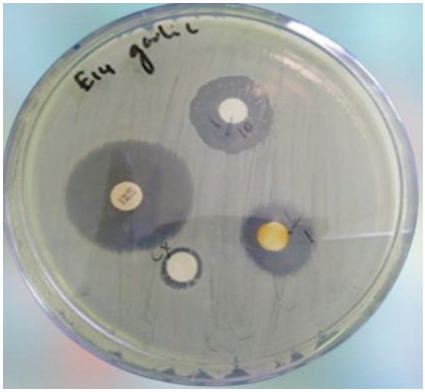


Foto 20: Aktiviti antimikrob yang sederhana daripada minyak pati komersial bawang putih terhadap *V.parahaemolyticus* AHPND patogen (E14)

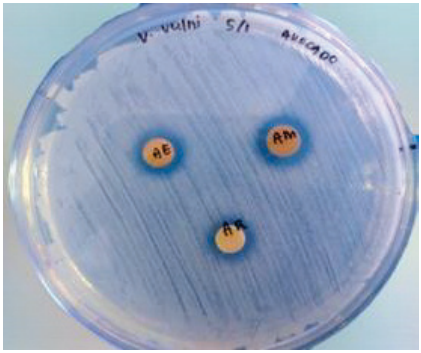


Foto 21: Aktiviti antimikrob yang rendah (DIZ ≤ 9 mm) daripada ekstrak biji avocado menggunakan pelarut etanol, metanol dan akueus (AE, AM, AR) terhadap patogen udang *V. vulnificus*

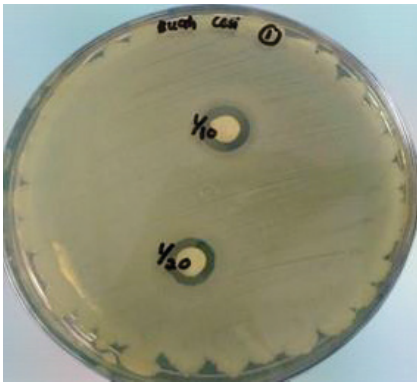


Foto 22: Aktiviti antimikrob yang rendah (DIZ ≤ 9 mm) daripada buah ceri terhadap *V. parahaemolyticus* (Vp14)

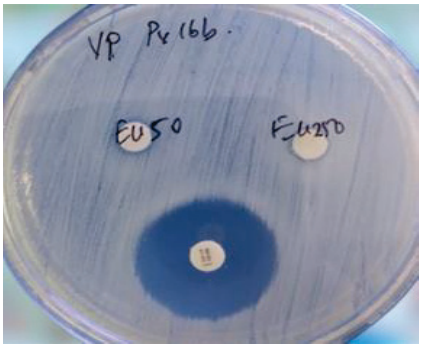


Foto 23: Aktiviti antimikrob yang sangat rendah daripada Eucabiotics (5% dan 2.5%) terhadap isolat *V. parahaemolyticus* dibandingkan dengan sensitiviti terhadap antibiotik tetracycline (30 ug/disc).

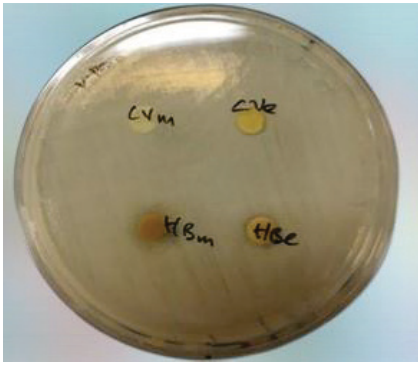


Foto 24. Tiada antimikrob aktiviti dari ekstrak metanol (CVm), ekstrak etanol (CVe) *C. vulgaris* dan DIZ yang sangat rendah dikesan daripada Hempedu Bumi (metanol, HBm) dan ekstrak etanol (BHe)

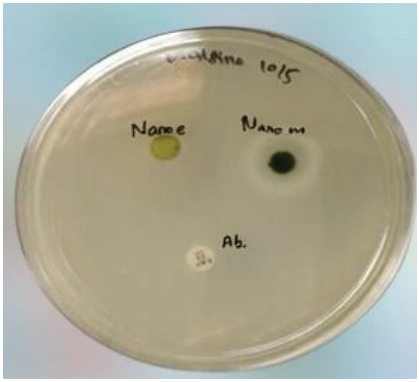


Foto 25: Aktiviti antimikrob yang rendah daripada ekstrak metanol mikroalga *Nannochloropsis* sp. terhadap *V. alginolyticus* dengan kerintangan isolat terhadap antibiotik ampicillin (AMP 30 ug/disc)

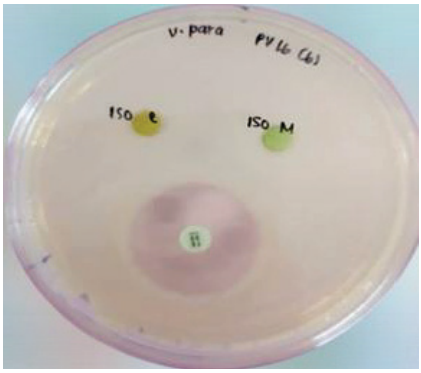


Foto 26: Tiada antimikrob aktiviti dikesan dari mikroalga *Isochrysis* sp. terhadap *V. parahaemolyticus* isolat PV16(b)

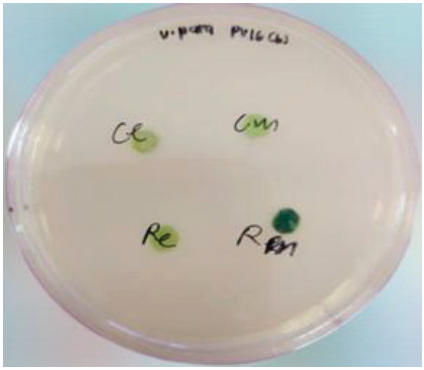


Foto 27: Tiada antimikrob aktiviti dikesan dari mikroalga *C. vulgaris* (telah didedahkan dengan faktor tekanan) ekstrak etanol (ce) dan metanol (cm) dan *Rhodomonas* sp. terhadap *V. parahaemolyticus* PV16(b)

Jadual 14. Ringkasan ujian saringan mengesan aktiviti antimikrob daripada ekstrak tumbuhan dan herba terhadap *V. parahaemolyticus* AHPND patogen menggunakan kaedah serapan cakera Kirby-Bauer yang dijalankan pada tahun 2017 sehingga 2021.

Tumbuhan/herba		Jenis ekstrak	Diameter zon rencatan (DIZ)	Antimikrob aktiviti	Isolat *V.parahaemolyticus*
Muntingia calabura Linn (Kerukup Siam/ Ceri Hutan)	 Buah	Metanol/ Etanol /Air	≤ 9 mm	Sangat Rendah	5
	 Daun	Metanol/ Etanol /Air	≤ 9-12 mm	Rendah	5
	 Bunga	Metanol/ Etanol /Air	≤ 9-12 mm	Rendah	5
Nannochloropsis sp. (Mikroalga)		Metanol/ Etanol /Air	≤ 9 mm	Sangat Rendah	2
Chlorella vulgaris (Mikroalga)		Metanol/ Etanol	≤ 9 mm	Sangat Rendah	1
Isochrysis sp. (Mikroalga)		Metanol/ Etanol	≤ 9 mm	Sangat Rendah	1
Rhodomonas sp. (Mikroalga)		Metanol/ Etanol	≤ 9 mm	Sangat Rendah	1
Piper betle (Sireh)		Air	>16 mm (22.2 ± 0.58)	Sederhana dan Tinggi	3
Eucalyptus oil, ≥ 5% (Eucabiotics)		Air	≤ 9 mm	Sangat Rendah	1

Tumbuhan/herba		Jenis ekstrak	Diameter zon rencatan (DIZ)	Antimikrobial aktiviti	Isolat *V.parahaemolyticus*
Persea american (Biji Avocado)		Metanol/ Etanol /Air	≤ 9 mm	Sangat Rendah	1
Phyllanthus amarus (Dukung anak)		Metanol/ Etanol /Air	≤ 9 mm	Sangat Rendah	1
Andrographis paniculata (Hempedu bumi /bitter king)		Metanol/ Etanol /Air	≤ 9 mm	Sangat Rendah	1
Citrus aurantiifolia (Minyak pati limau)		produk komersil	11 – 26 mm (19.0 ± 2.7)	Rendah, Sederhana dan Tinggi	10
Citrus limon (Minyak pati lemon)		produk komersil	7 - 23 mm (16.2 ± 3.9)	Rendah, Sederhana dan Tinggi	10
Cinnamomum zeylanicum (Minyak pati kayu manis)		produk komersil	8 – 23 mm (17.4 ± 3.9)	Rendah, Sederhana dan Tinggi	10
Allium sativum (Minyak pati bawang putih)		produk komersil	7 – 14 mm (10.2 ± 2.1)	Rendah dan Sederhana	10
Cymbopogon nardus (Minyak pati serai wangi)		produk komersil	4 – 14 mm (9.9 ± 2.9)	Rendah dan Sederhana	10

5.5 Surfaktan ester lipid sebagai rawatan AHPND

Udang putih yang menerima makanan pelet bercampur surfaktan ester lipid menunjukkan kadar kemandirian yang lebih baik iaitu 67 - 72% berbanding dengan udang yang menerima makanan pelet biasa, 0 - 58%. Kadar piawai tumbesaran udang berjulat 21.9 - 24.5% pada udang yang menerima rawatan berbanding dengan hanya 22.8% pada udang dalam tangki kawalan (Jadual 15). Kehadiran bakteria kumpulan *Vibrio* sebelum dan selepas rawatan direkodkan bagi semua udang kajian samada dari eksperimen pertama atau kedua. Walau bagaimanapun, pemeriksaan patologi menunjukkan tiada penyingkiran sel-sel epitelium dari hepatopankreas tubul pada semua udang putih dari kedua-dua eksperimen tersebut (Jadual 16).

Jadual 15: Perbandingan tumbesaran udang putih positif AHPND selepas dirawat selama 42 hari dengan surfaktan ester lipid di makmal basah

Tangki	**Berat awal (g)**	**Berat akhir (g)***	**Peningkatan berat (g per hari) ****	**Kadar piawai tumbesaran (%)*****
Eksperimen Pertama				
a) Tangki kawalan	1.4 ± 2.0	udang mati	Tiada data	Tiada data
b) Tangki rawatan	1.4 ± 2.0	10.6 ± 1.1	0.2	21.9
Eksperimen Kedua				
a) Tangki kawalan	1.4 ± 2.0	11.0± 4.6	0.2	22.8
b) Tangki rawatan	2.5 ± 2.0	14.0 ± 1.9	0.2	24.5

Nota: *selepas 42 hari rawatan akhir, **[(Akhir –Awal)/ hari], ***[(Akhir–awal/hari) x100%]

Jadual 16: Perbandingan kadar kemandirian udang putih ternak positif AHPND selepas dirawat selama 42 hari dengan surfaktan ester lipid di makmal basah

Rawatan udang putih ternak selepas 42 hari	**Eksperimen Pertama**		**Eksperimen kedua**	
	Tangki Rawatan	**Tangki Kawalan**	**Tangki Rawatan**	**Tangki Kawalan**
Kadar kemandirian (%)	67	0.0	72	58
Kehadiran jangkitan bakteria	*V. parahaemolyticus* *V. fluvialis*	*V. parahaemolyticus*	*V. alginolyticus*	*V. alginolyticus*
Patologi AHPND	Tiada penyingkiran sel di tubul hepatopankreas	Penyingkiran sel di tubul hepatopankreas	Tiada penyingkiran sel di tubul hepatopankreas	Penyingkiran sel di tubul hepatopankreas

5.6 Ujian cabaran keatas udang putih yang diberi diet EOCIN

Eksperimen di makmal bagi ujian cabaran yang dijalankan ini menunjukkan pemberian makanan pelet bercampur EOCIN pada regim 1.5% (isipadu/berat pelet makanan) selama 14 hari berturut-turut bermula dari peringkat PL apabila udang dimasukkan ke tangki ternakan sehingga usia ternakan mencapai hari ke-30 (DOC 30) didapati tidak menunjukkan perbezaan yang signifikan dengan kumpulan udang kawalan apabila dicabar dengan *V. parahaemolyticus*. Kematian 100% direkodkan daripada kedua-dua kumpulan selepas hari ke-3 ujian cabaran. Walaubagaimanapun, ujian PCR daripada sampel udang yang dijangkiti menunjukkan kehadiran plasmid tanpa *PirA/B* toksin gen daripada hepatopankreas udang putih yang menerima makanan bercampur EOCIN. Tanda patologi yang dilihat daripada pemeriksaan udang adalah organ hepatopankreas yang pucat dan kemerahan pada lapisan luar kulit terutamanya di bahagian abdominal segmen dan ekor. Kajian lanjut yang lebih mendalam perlu dijalankan untuk mengesahkan keberkesanan EOCIN dalam menghilangkan keupayaan/virulen bakteria *V. parahaemolyticus* untuk menghasilkan *PirA/B* toksin.

5.7 Aplikasi EOCIN dalam diet ternakan udang putih sebagai kawalan AHPND di lapangan

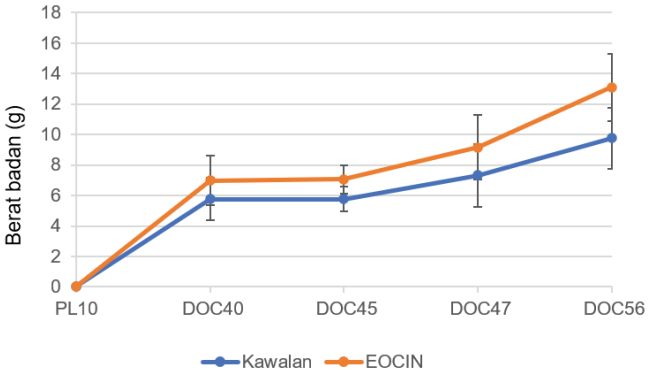
Sebanyak dua pusingan ternakan berkapasiti tinggi telah dijalankan bersama dengan syarikat tempatan di lokasi Teluk Tempoyak, Pulau Pinang. Aplikasi EOCIN bercampur dengan makanan pelet biasa dalam pusingan ternakan pertama bermula dari 4 Disember 2020 hingga 27 Januari 2021. Kajian ini melibatkan satu pusingan ternakan sehingga hasil dituai bermula apabila benih udang di bawa masuk ke tangki ternakan sehingga penamatan dua kali regim dos rawatan EOCIN selesai. Persampelan bermula daripada peringkat pasca-larva (PL10) apabila benih udang dimasukkan ke tangki ternakan dengan kadar kepadatan stok 350 ekor/m^3 dan diulang pada DOC 40, 45, 47 dan 56 untuk melihat pertumbuhan berat badan udang putih selepas tamat regim rawatan EOCIN. Keputusan pusingan ternakan pertama menunjukkan udang putih yang menerima pemberian makanan bercampur EOCIN mempunyai pertumbuhan berat badan yang lebih tinggi daripada udang yang menerima makanan pelet biasa (Rajah 14 dan 15). Saringan AHPND dan EHP di peringkat benih dan di sepanjang kajian dijalankan adalah negatif.

Kajian yang sama diulang bagi satu pusingan ternakan lagi yang bermula daripada 23 Februari 2021 sehingga 11 Mei 2021. Empat tangki ternakan udang putih dengan kapasiti 300,000 ekor udang/tangki dan kadar kepadatan 350 ekor/m^3 telah digunakan dan dua tangki menerima makanan bercampur EOCIN dan hanya satu tangki kawalan. Ujian saringan awal dari setiap tangki eksperimen telah dijalankan secara berkala sehingga tamat rawatan. Ujian PCR ke atas AHPND menunjukkan semua sampel adalah negatif. Namun, kesemua udang dari tangki EOCIN dan kawalan didapati positif keatas penyakit lain iaitu penyakit serangan mikrospora *Enterocytozoon hepatopenaei* (EHP) dengan julat prevalen 25 hingga 95%. Tiada perbezaan yang ketara dari segi pertumbuhan udang samada berat badan dan panjang (Rajah 16 dan 17). Jangkitan EHP

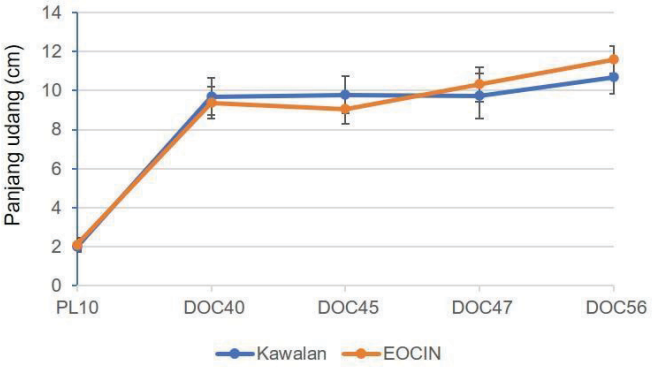
seringkali dikaitkan dengan tumbesaran yang terbantut (Chayaburakul et al., 2014; Kmmari et al., 2018 & Wan Muhd et al., 2021)

Walaupun, tiada AHPND dikesan sepanjang kajian dijalankan, plasmid (5%) daripada tisu udang (hepatopankreas) dan beberapa isolat bakteria *V. parahaemolyticus* (7.7%) dikesan membawa plasmid tanpa toksin gen *PirA/B* (Jadual 17). Hanya virulen strain daripada *V. parahaemolyticus* diketahui membawa plasmid pVA1 (63-70 kb) enkoding binari toksin *PirA/B* dan mempunyai kapasiti untuk menghasilkan toksin menyebabkan penyakit (Dong et al., 2017). Plasmid pVA1 ini juga membawa kluster gen yang dikaitkan dengan pemindahan konjugatif (Kumar et al., 2020). Strain pVA1 juga membawa gen pemindahan konjugatif dan mobilisasi plasmid yang membolehkan plasmid membawa toksin *PirA/B* berpindah di antara *Vibrio* spp. seperti *V. harveyi*, *V. campbellii* (Kondo et al., 2015), *V. owensii* (Liu et al., 2018) dan ke bakteria spesis lain (Xiao et al., 2017; Han et al., 2017). Selain daripada itu, di dalam keadaan 'stress' atau situasi yang kondusif, pertumbuhan bilangan koloni bakteria *V. parahaemolyticus* boleh meningkat sehingga mencapai infektif dos (10^3 cfu/mL) melalui proses penghasilan biofilm dan mekanisma quorum sensing di antara sel bakteria menyebabkan terhasilnya toksin *PirA/B* (Restrepo et al. 2016; Phiwsaiya et al. 2017). Oleh itu, isolat *V. parahaemolyticus* membawa plasmid masih mempunyai potensi untuk menjadi virulen terutamanya apabila bakteria sel yang tinggi dikesan dalam tisu udang (50-70%) (Rajah 18). Terdapat banyak faktor lain yang diketahui boleh mempengaruhi pertumbuhan bakteria di dalam sistem akuatik terutamanya kualiti air seperti saliniti, suhu dan kandungan nutrien.

Analisis perbandingan prevalen bakteria pada udang putih menerima EOCIN dan pelet makanan biasa menunjukkan *V. parahaemolyticus* dan *V. vulnificus* mendominasi bakteria lain yang diasingkan daripada organ hepatopankreas. Pemberian makanan bercampur EOCIN tidak menghasilkan perbezaan kepada populasi mikrob daripada organ pencernaan udang jika dibandingkan dengan kumpulan tanpa EOCIN (Rajah 19 dan 20), secara tidak langsung situasi ini menunjukkan kehadiran patogen *V. parahaemolyticus* yang tinggi di dalam sistem akuatik. Pelbagai faktor terutamanya kualiti air dan pengurusan kolam/tangki ternakan mempengaruhi pertumbuhan mikrob atau patogen di dalam kolam ternakan. Walaubagaimanapun, pemberian EOCIN didapati memberi kesan positif di dalam meningkatkan kadar pertumbuhan udang dan imunisasi terhadap penyakit AHPND. Prevalen *V. parahaemolyticus* adalah agak tinggi (15 - 70%) dengan pengesanan plasmid sebanyak 7.7% pada udang putih, walaubagaimanapun ujian PCR bagi AHPND (pengesanan toksin gen *PirA/B*) adalah negatif. Tiada masalah penyakit dikesan dan kematian tinggi tidak dilaporkan oleh penternak. Kajian ini menunjukkan pemberian EOCIN boleh meningkatkan pertumbuhan udang dari segi peningkatan berat badan yang signifikan pada udang putih yang menerima diet EOCIN berbanding dengan diet pelet biasa.



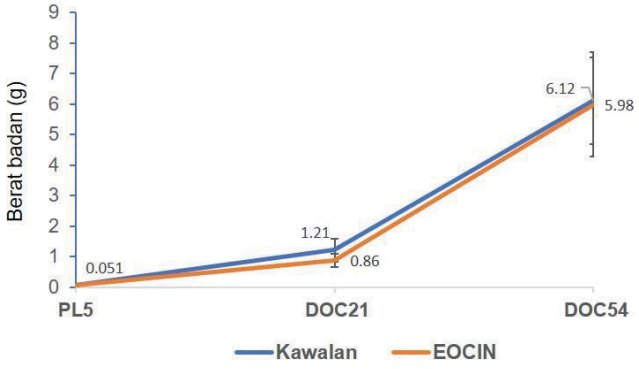
Rajah 14. Perbandingan pertumbuhan berat badan (g) udang putih yang menerima diet EOCIN dan kawalan dalam percubaan pertama di lapangan.



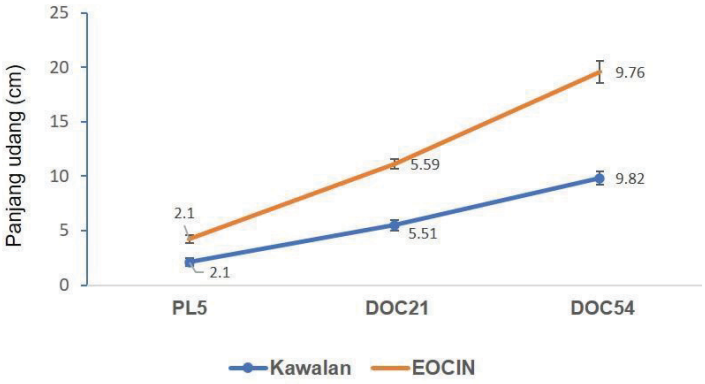
Rajah 15. Perbandingan pertumbuhan panjang (cm) udang putih dari tangki kawalan dan EOCIN bagi percubaan pertama di lapangan.

Jadual 17: Keputusan prevalen AHPND dan bakteria *V. parahaemolyticus* (%) sepanjang persampelan udang putih bagi kajian EOCIN dalam percubaan ke-2.

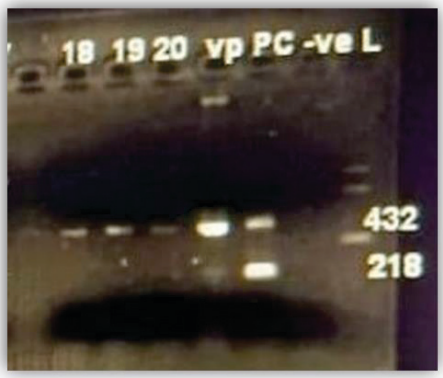
	Hepatopankreas udang (sampel postif/jumlah sampel)		***V. parahaemolyticus* (n)** (isolat postif/jumlah isolat)	
	Pir A/B	Plasmid	*Pir A/B*	Plasmid
Status PL sebelum kajian DOC 6	- ve (0/10)	- ve (0/10)	Tiada isolat	Tiada isolat
Prevalen AHPND(%) PL	**0**	**0**	**-**	**-**
Status udang semasa kajian DOC 21				
Tangki Kawalan	-ve (0/10)	-ve (0/10)	-ve (0/10)	-ve (0/10)
Prevalen AHPND(%) Tangki Kawalan	**0**	**0**	**0**	**0**
Tangki EOCIN - Replikat 1	-ve (0/10)	-ve (0/10)	Tiada isolat	Tiada isolat
Tangki EOCIN - Replikat 2	-ve (0/10)	-ve (0/10)	-ve (0/2)	-ve (0/2)
Purata Prevalen AHPND(%) Tangki EOCIN	**0**	**0**	**0**	**0**
Status udang semasa kajian DOC 54				
Tangki Kawalan	-ve (0/10)	-ve (0/10)	-ve (0/10)	-ve (0/8)
Prevalen AHPND(%) Tangki Kawalan	**0**	**0**	**0**	**0**
Tangki EOCIN - Replikat 1	-ve (0/10)	-ve (0/10)	-ve (0/8)	+ve (1/8)
Tangki EOCIN - Replikat 2	-ve (0/10)	+ve (1/10)	-ve (0/5)	-ve (0/5)
Purata Prevalen AHPND(%) Tangki EOCIN	**0**	**5 (Plasmid)**	**0**	**7.7 (Plasmid)**



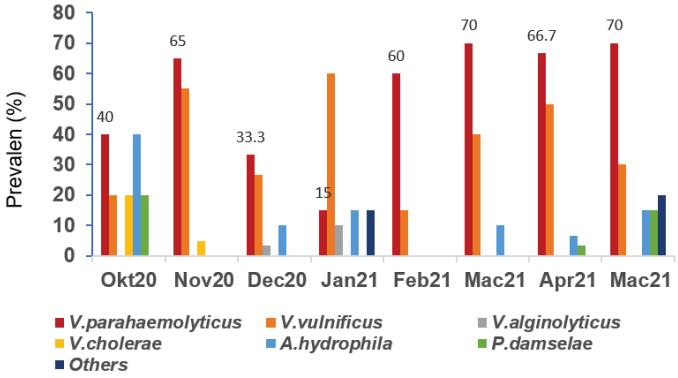
Rajah 16. Perbandingan pertumbuhan berat badan (g) udang putih menerima diet EOCIN dengan kawalan semasa percubaan kedua di lapangan.



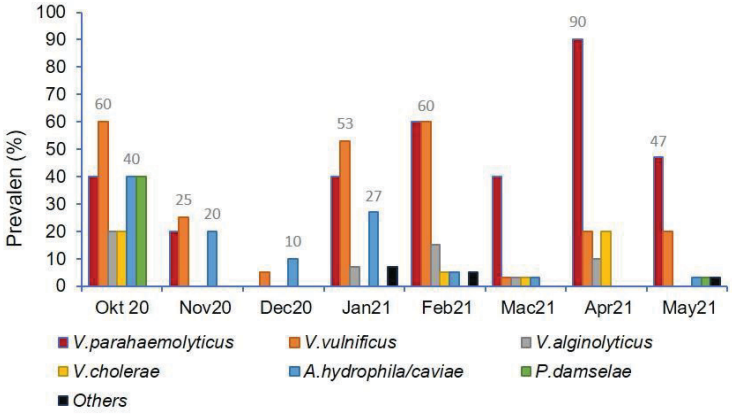
Rajah 17. Kawalan semasa percubaan kedua di lapangan



Rajah 18. Gel elektroforesis menunjukkan pengesanan kehadiran plasmid (432 bp) pada bakteria *V. parahaemolyticus* tanpa *PirA/B* toksin gen dipencilkan daripada tisu hepatopankreas udang



Rajah 19: Prevalen bakteria *V. parahaemolyticus* yang dipencilkan daripada hepatopankreas udang putih menerima makanan bercampur EOCIN.



Rajah 20: Prevalen bakteria daripada hepatopankreas udang putih yang menerima makanan pelet biasa.

5.8 Pengesanan AHPND dengan skor kad usus udang

Percubaan pengesahan hubungan antara kehadiran sel epitelium tersingkir dengan jangkitan AHPND di makmal menunjukkan udang yang dicabar dengan 1.0 x 10^8 CFU/ml *V. parahaemolyticus* mula mati selepas 6 jam pertama dengan kadar kematian sebanyak 10%. peratus kematian dan mula meningkat kepada 43% selepas 24 jam (Rajah 21). Kehadiran sel epitelium tersingkir di usus mula dikesan selepas 24 jam dengan prevalen 67%. Pengesanan AHPND juga dikesan selepas 24 jam dengan kaedah histologi (Foto 28). Perbandingan kehadiran sel epitelium hepatopankreas di antara udang yang dicabar dengan *V. parahaemolyticus* dan kawalan menunjukan perbezaan yang signifikan ($p<0.05$). Jangkitan AHPND menyebabkan inflamasi dan nekrosis pada sel hepatopankreas di tubul organ hepatopankreas udang menyebabkan sel epitelium hepatopankreas tersingkir ke lumen tubul dan seterusnya dikesan di usus selepas 24 jam (Foto 29 - 30).

Keputusan di makmal ini seterusnya diuji di peringkat lapangan. Sebanyak 347 kolam ternakan udang putih dari negeri Kedah dan Terengganu telah dikenalpasti. Perbandingan sebanyak 1580 ekor udang putih daripada sampel yang dijangkiti, tidak dijangkiti dan tidak diketahui status penyakitnya telah dilakukan. Kehadiran sel epitelium hepatopankreas tersingkir di usus menunjukkan skor di antara 0 hingga 4. Penilaian semua skor yang diperolehi daripada usus juga dibandingkan dengan kehadiran patologi positif AHPND atau kehadiran positif bakteria *V. parahaemolyticus*. Hasil kajian

menunjukkan bahawa 0% (0/40) atau tiada sel-sel hepatopankreas diperhatikan dalam usus pada udang sihat berbanding usus udang berpenyakit yang menunjukkan 40% (12/30) kehadiran sel-sel epitelium hepatopankreas di usus (Jadual 18). Kehadiran sel epitelium hepatopankreas bagi sampel udang yang tidak diketahui status penyakitnya juga menunjukkan 52% (90/172) untuk udang dari Kedah. Peratusan sebanyak 77% (69/90) juga turut dikesan pada udang dari Terengganu. Skor kad usus menunjukkan skala 0 bagi udang sihat manakala skala yang lebih tinggi iaitu skala 1, 2 dan 4 bagi udang berpenyakit yang telah disahkan positif AHPND. Bagi udang dari Kedah dan Terengganu, skor kad usus berada dalam julat skala 0 hingga 4. Skor kad usus dengan skala 2, 3 dan 4 menunjukkan kehadiran positif bakteria *V. parahaemolyticus* manakala skala 1, 2 , 3 dan 4 untuk bakteria lain seperti *V. alginolyticus, V. vulnificus* dan *Aeromonas hydrophila*. Skala skor kad usus 1, 2, 3 dan 4 menunjukkan perubahan patologi seperti kehadiran nukleus yang besar dan penyusupan sel darah hemosit ('hemocyte').

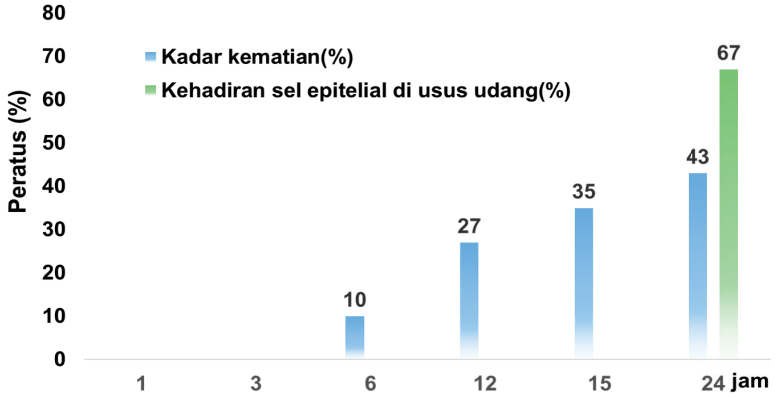
Kajian yang sama telah diulang di kolam udang putih berskala besar di lokasi Perak. Pengambilan sampel untuk perbandingan menggunakan kaedah skor kad usus, bakteriologi, histologi dan PCR bermula dari DOC 15 hingga 30 di empat kolam. Hasil kajian menunjukkan kehadiran agen penyebab AHPND iaitu bakteria *V. parahaemolyticus* seawal tempoh ternakan 15 hari di dalam kolam (Jadual 19). Penemuan ini disokong oleh analisis PCR menggunakan kit IQ2000 ems2 yang menunjukkan hasil positif AHPND dan analisis histopatologi sampel usus udang yang menunjukkan penyingkiran sel hepatopankreas di lumen tubul dan juga di usus udang. Bacaan skor kad usus juga didapati meningkat sehingga ke skor 4 pada akhir kajian berbanding skor 1 - 2 pada awal kajian (Jadual 19). Perbandingan skor kad dengan keputusan positif PCR menunjukkan ianya boleh dijadikan indikator penyakit AHPND untuk pengesanan awal di peringkat ladang; sekaligus memungkinkan langkah mitigasi awal diambil oleh penternak bagi membendung kejadian wabak AHPND.

Analisis ANOVA sehala menunjukkan perbezaan signifikan dikesan pada skor 0 dengan skor 1, 2, 3 dan 4. Keputusan ini seterusnya menunjukkan kaedah skor kad usus boleh digunakan untuk membantu dalam diagnosis EMS/AHPND sebagai langkah awal di peringkat ladang. Namun, bacaan skor perlu diperhalusi agar aplikasi kaedah ini lebih mudah dan praktikal kepada penternak. Pengubahsuaian telah dilakukan ke atas bacaan skor secara keseluruhan dari setiap populasi udang dan juga bilangan udang yang perlu disampel. Bacaan skor dikurangkan kepada hanya 3 bacaan saja iaitu skor 0, 0 & 1 dan 0,1 & 2. Bacaan skor perlu berdasarkan kepada penyampelan minima lima ekor udang dari setiap kolam ternakan. Interpretasi skor keseluruhan seterusnya disusuli dengan beberapa tindakan segera yang perlu diambil oleh penternak. Kehadiran skor 0 diinterpretasi sebagai udang yang sihat, skor 0 & 1 menunjukkan udang tidak sihat dan memerlukan tindakan seperti pengurangan kadar pelepasan/kepadatan, penukaran air atau sampel perlu dihantar ke makmal untuk pengesahan penyakit udang. Kehadiran skor 0, 1 & 2 merujuk kepada udang yang tidak sihat dan memerlukan tindakan yang cepat kerana udang dijangka akan mengalami kematian. Antara contoh tindakan segera yang perlu diambil ialah melakukan beberapa kali tuaian hasil ternakan.

Keputusan pengubahsuaian skor kad usus ini telah diuji dan pengesahan AHPND turut dibandingkan dengan kaedah PCR. Perbandingan ketepatan pengesanan AHPND dalam 244 ekor udang menunjukkan peratus ketepatan kaedah penilaian melalui skor kad usus adalah 95 - 98% manakala 100% bagi pengesanan kaedah PCR (Jadual 20). Perbezaan pengesanan AHPND di antara kaedah PCR dan skor kad usus adalah antara 2% bagi udang yang tidak sihat dan 5% bagi udang yang sihat. Hasil keputusan ini seterusnya membawa kepada penggantian nama kaedah skor kad usus kepada `SHOS-Spotter' yang bermaksud pengesanan kesihatan udang secara in situ.

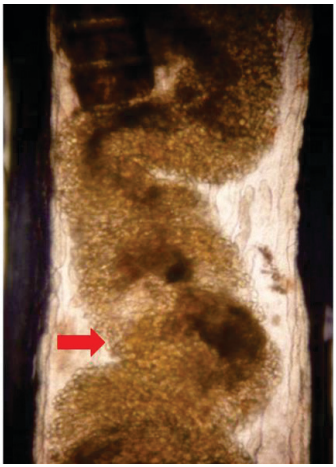
Kajian perbandingan ini diulang di tiga ladang udang di lapangan yang mengalami kematian awal di usia ternakan dengan menambah satu lagi perbandingan pengesanan AHPND iaitu kaedah histologi. Keputusan kajian menunjukkan penggunaan kaedah SHOS-Spotter boleh mengesan kejadian AHPND dengan purata ketepatan 95% berbanding dengan 100% bagi kaedah histologi dan PCR (Jadual 21).

Pengesahan pengubahsuaian SHOS-Spotter turut direkod dalam pemantauan satu pusingan ternakan udang putih dalam kolam yang disahkan positif AHPND pada peringkat PL di Kedah. Penternak dinasihatkan mengambil tindakan berdasarkan interpretasi skor kad usus seperti mengurangkan kadar pelepasan dari 150 ekor/m^3 kepada 80 ekor sekiranya kehadiran skor 0 & 1. Keputusan percubaan di lapangan oleh penternak menunjukkan secara purata kadar kemandirian bagi udang yang positif AHPND sehingga DOC 100 adalah 58% (Rajah 22). Purata berat yang diperolehi adalah 13 g dimana nilai ini termasuk dalam julat tumbesaran udang putih bagi tempoh ternakan kurang dari DOC 100. Secara purata, kehadiran sel epitelium hepatopankreas di usus adalah 49%.



Rajah 21: Peratus kematian dan kehadiran sel epitelium di usus udang dalam tempoh 24 jam selepas dicabar dengan *V. parahaemolyticus*

Usus tanpa sel epitelium hepatopankreas yang tersingkir



Usus dengan sel epitelium hepatopankreas yang tersingkir (anak panah)

Foto 28: Kehadiran sel epitelium hepatopankreas di usus udang (anak panah) selepas 24 jam di bawah pemerhatian mikroskop.

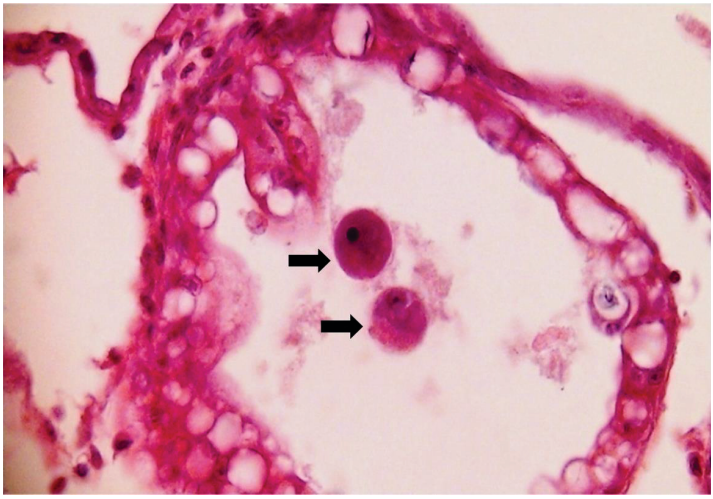


Foto 29: Penyingkiran sel epitelium hepatopankreas di lumen tubul (anak panah) dan merupakan pengesahan patologi AHPND melalui kaedah histologi. H&E

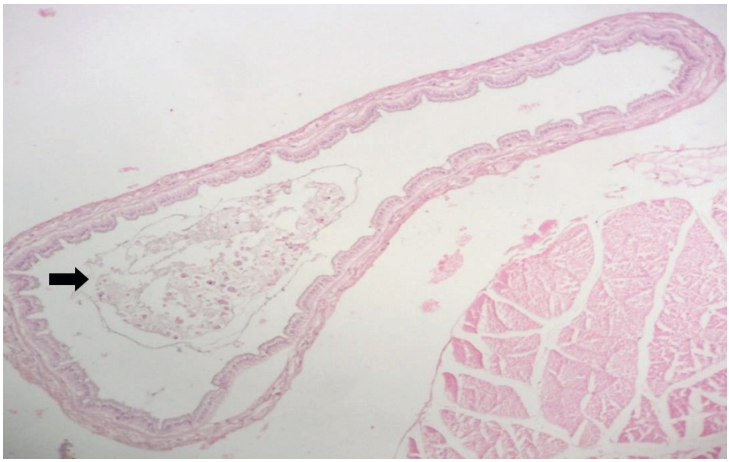


Foto 30: Kehadiran sel epitelium hepatopankreas (anak panah) di usus udang selepas 24 jam dicabar dengan *V. parahaemolyticus* Kaedah histologi, H&E.

Jadual 18: Perbandingan pengesanan AHPND di antara kaedah skod kad usus, kehadiran bakteria *V. parahaemolyticus* dan analisis patologi AHPND bagi sampel udang yang tidak diketahui status dari Kedah dan Terengganu.

	Bil. udang diperiksa	Kehadiran sel epitelium di usus (%)	Skor Kad usus				
			0	1	2	3	4
Pemerhatian sel HP yang terselingkir di usus di bawah mikroskop cahaya							
Udang sihat	40	0(0)	40	0	0	0	0
Udang tidak sihat	35	12(34.3)	23	2	4	0	6
Status tidak diketahui dari Kedah	172	90 (52)	82	37	30	9	14
Status tidak diketahui dari Terengganu	90	69(77)	21	35	15	11	8
Analisis kehadiran bakteria							
V.parahaemolyticus			-	-	+	+	+
Bakteria lain			+	+	+	+	+
Analisis patologi AHPND							
Karyomegaly			+	+	+	+	+
Penyingkiran sel HP			-	+	+	+	+
Hemocytic infiltration			+	+	+	+	+
Encapsulated/melanized			+	+	+	+	+

Jadual 19: Perbandingan pengesanan AHPND di antara kaedah skor kad usus, kehadiran bakteria *V. parahaemolyticus,* ujian PCR dan analisis patologi AHPND bagi sampel udang dari satu tempoh ternakan bermula dari DOC 15 - 78 dan DOC 30 - 87.

1. Tempoh ternakan bermula dari DOC 15 - 78

	DOC 15	DOC 30	DOC 50	DOC 65	DOC 78
Julat berat(g)	1	1 - 2.2	2.9 - 3.9	7.9 – 10.4	7.6 – 11.3
Skor kad usus					
0 (%)	30 - 40	40 - 80			
1 (%)			40 - 55	80	20 - 40
2 (%)			40 - 55	40 - 80	40
3 (%)			55	40 - 80	40
4 (%)				40 - 80	20 - 40
Kehadiran bakteria *V. parahaemolyticus*	+	+	+	+	+
PCR ems2 IQ2000	+	+	+	+	+
Analisis patologi AHPND					
Penyingkiran sel epitelium di tubul HP (%)	20		50 - 60	-	20 - 50
Hemocytic infiltration (%)	60		40 - 83	-	75 - 100
Encapsulated/melanized (%)		20	20 - 33	-	20 - 50

2. Tempoh ternakan bermula dari DOC 30 - 87

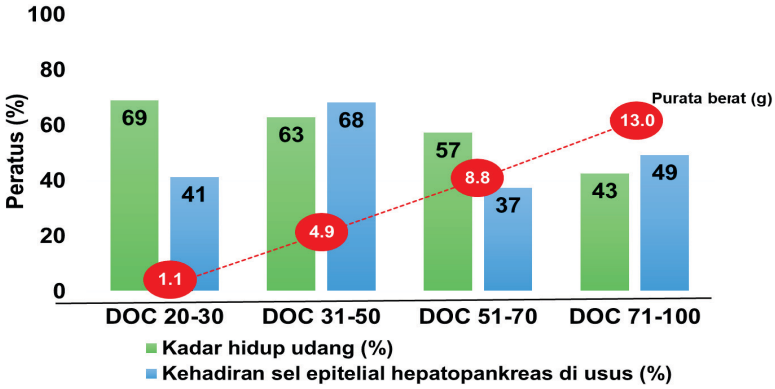
	DOC 30	DOC 52	DOC 72	DOC 87
Julat berat(g)	2.5 - 2.8	5.4 - 6.1	5.1 - 7.2	10.9 - 11.5
Skor kad usus				
0 (%)	0	0	0	0
1 (%)	70	40 - 60	20 - 40	40
2 (%)	70	40 - 60	40	10
3 (%)	0	40 - 60	0	40
4 (%)	0	60	20 - 40	40
Kehadiran bakteria *V. parahaemolyticus*	+	+	+	+
PCR ems2 IQ2000	+	+	+	+
Analisis patologi AHPND				
Penyingkiran sel epitelium di tubul HP (%)	20	20 - 40	100	20 - 80
Hemocytic infiltration (%)		60	17 - 50	40
Encapsulated/melanized (%)			80	20 - 40

Jadual 20: Perbandingan ketepatan pengesanan AHPND dengan kaedah PCR dan pengubahsuaian penilaian skor kad usus.

Kaedah PCR				Kaedah Skor Kad Usus				Perbezaan kaedah PCR dengan Skor Kad Usus (%)
Alat PCR	Bilangan udang	Peratus (%)	Status kesihatan	Skor	Bilangan udang	Peratus (%)	Status kesihatan	
Negatif	170	70%	-ve AHPND	0	158	65	Sihat	5%
Positif	47	19%	+ve AHPND	0 & 1	53	22	Tidak Sihat	2%
Positif	27	11%	+ve AHPND	0, 1 & 2	33	14	Tidak Sihat	2%

Jadual 21: Perbandingan kaedah histologi, PCR dan penilaian skor kad usus (SHOS-Spotter) di tiga ladang udang di Kuching, Sarawak.

Pengesanan AHPND	Udang +ve AHPND/jumlah udang (%)			
	Ladang 1	Ladang 2	Ladang 3	Purata
Kaedah Histologi	25/25 (100)	85/85 (100)	15/15 (100)	**100**
PCR	50/50 (100)	130/130 (100)	50/50 (100)	**100**
SHOS-Spotter	50/50 (100)	110/130 (84.6)	50/50 (100)	**95**



Rajah 22: Perhubungan di antara kehadiran sel epitelium hepatopankreas di usus dengan kadar kemandirian udang ternak yang menggunakan PL positif AHPND sepanjang tempoh satu pusingan ternakan di ladang.

6.0

OUTPUT KAJIAN

a). Status kejadian wabak penyakit pada tahun 2011 telah dikenal pasti berpunca dari serangan bakteria *V. parahaemolyticus* pencetus toksin yang dikenali sebagai *Acute Hepatopancreatic Necrosis Disease* (AHPND). Penyakit ini dikategorikan sebagai penyakit kemunculan baru di Malaysia dan pengesahan AHPND membolehkan pelaporan kemunculan penyakit baru di peringkat kebangsaan dan antarabangsa.

b). Impak AHPND menyebabkan penurunan pengeluaran udang putih pada 2011 berbanding dengan 2010. Anggaran kerugian penurunan statistik pengeluaran udang ternak putih dianggar USD 0.1billion pada tahun 2011. Kerugian akibat AHPND turut direkodkan dalam pelaporan kes diagnostik di makmal NaFisH semasa tahun 2011 hingga 2022 adalah berjumlah RM3,041,775. Nilai ini adalah bagi ternakan di tangki atau kolam bermula dari tempoh ternakan berumur satu hari sehingga mencapai saiz pasaran.

c). Saringan bahan mesra alam menunjukkan produk hasilan serai, minyak pati komersil kayu manis, lemon dan limau nipis boleh bertindak sebagai perencat kepada bakteria pencetus AHPND. Justeru itu, produk ini boleh digunakan sebagai kawalan dan rawatan ke atas jangkitan bakteria pencetus AHPND.

d). Aplikasi minyak pati komersil kayu manis berpotensi untuk dijadikan sebagai kawalan untuk mengurangkan peratus kehadiran toksin pada bakteria *V. parahaemolyticus* penyebab AHPND disamping sebagai pencetus selera makan untuk tumbesaran udang ternak.

e). Aplikasi bahan lipid ester berpotensi untuk dijadikan sebagai rawatan alternatif untuk mengurangkan peratus kematian udang yang telah dijangkiti AHPND.

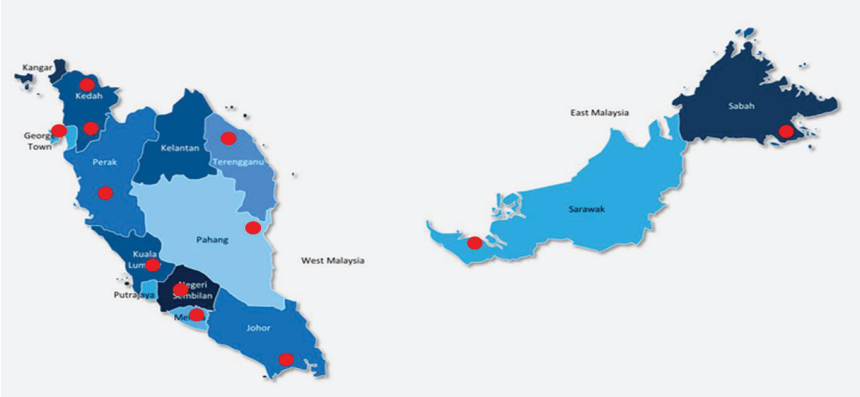
f). Satu kaedah pengesahan AHPND berdasarkan skor kad kehadiran sel epitelium hepatopankreas di usus telah berjaya dibangunkan. Skor kad antara udang yang dijangkiti AHPND dengan udang tanpa jangkitan dapat dibezakan dari segi patologi dan kehadiran bakteria *V. parahaemolyticus* di organ hepatopankreas. Hasil kajian diperhalusi dan berjaya menghasilkan kaedah pengesanan awal AHPND yang diberi nama SHOS-Spotter. Kaedah ini lebih mudah, cepat dan praktikal kepada penternak dan telah berjaya di uji di makmal dan lapangan. Kaedah ini turut didaftar sebagai cap dagang di bawah MyIPO.

7.0

KESIMPULAN

Kemunculan penyakit baru AHPND disahkan etiologinya pada tahun 2011 di Malaysia. Dalam tempoh 2011 hingga 2015, impak kerugian hasil dari pelaporan kes diagnostik di makmal NaFisH adalah berjumlah RM3,041,775 bagi ternakan di tangki dan kolam. Nilai impak ini adalah kecil berbanding dengan nilai pengurangan pengeluaran udang ternak sebenar disebabkan oleh ketiadaan maklumat lengkap. Perbandingan hasil pengeluaran udang pada 2010 dan 2011 menunjukkan penurunan sebanyak 19,729MT dengan nilai anggaran kerugian harga borong RM290, 364 260.

Selepas cetusan wabak penyakit AHPND pada tahun 2011, industri ternakan udang di Malaysia masih lagi mengalami wabak jangkitan AHPND dari masa ke semasa, namun pelaporan kes diagnostik semakin berkurangan dan sehingga 2021, prevalen jangkitan AHPND adalah rendah iaitu dibawah 23% (Rajah 23).



Rajah 23: Lokasi negeri di Malaysia yang masih dikesan positif AHPND pada tahun 2019.

Strategi pengurusan kolam dan ternakan udang di dalam mengawal pertumbuhan bakteria pencetus AHPND dilihat sebagai kunci utama di dalam mengurangkan risiko kepada cetusan penyakit EMS/AHPND. Penternak sering disarankan dengan penggunaan PL yang bebas penyakit dan melakukan prosedur kuarantin semasa kemasukan udang di peringkat nurseri di tangki atau kolam ternakan. Selain itu, ujian kesihatan seperti pemeriksaan kehadiran koloni *Vibrio* spp. terutamanya *V. parahaemolyticus* atau ujian tekanan 'stress test' ke atas saliniti turut disyorkan. Namun,

menempatkan pasca-larva udang ternakan dalam sistem kolam atau tangki ternakan yang mengandungi bakteria *V. parahaemolyticus* dengan prevalen yang tinggi (>50%) dipercayai meningkatkan risiko pembawa kepada cetusan penyakit AHPND.

Beberapa mekanisma telah dikenalpasti berupaya merencat regulasi gen virulen daripada bakteria patogen (Natrah et al., 2011). Penggunaan tumbuhan dan herbal serta penggunaan beberapa spesis mikroalga air hijau dikatakan mampu mengawal pertumbuhan populasi bakteria pencetus AHPND (Kokou et al., 2012). Fitokimia tumbuhan merupakan suatu sumber berpotensi bagi mencari kompoun bioaktif antimikrob. Kajian saintifik menunjukkan ekstrak tumbuhan herba seperti serai, minyak pati komersial kulit kayu manis (EOCIN), lemon dan limau nipis diketahui memberi kesan positif ke atas kesihatan haiwan akuatik dengan meningkatkan pertumbuhan, imunisasi dan rintangan terhadap penyakit.

Minyak pati kayu manis (EOCIN) mengandungi bahan aktif seperti anti-bakteria, anti-oksidan, anti-virus dan lain-lain. Pemberian makanan bercampur EOCIN menunjukkan mampu mengawal kejadian AHPND disamping meningkatkan kadar tumbesaran udang dan mengurangkan jurang kewujudan kepelbagaian saiz udang ternakan. Aplikasi ini seterusnya boleh membantu penternak dalam meningkatkan hasil pengeluaran di samping bertindak sebagai pencetus selera makan udang ternakan. Selain itu, kajian aplikasi rawatan ke atas udang selepas diserang AHPND dengan menggunakan surfaktan lipid ester menunjukkan ia mampu mengurangkan kejadian penyingkiran sel epitelium di tubul hepatopankreas. Aplikasi lipid ester turut berpotensi digunakan sebagai rawatan pasca-AHPND dalam mengurangkan peratus kematian. Maklumat ini perlu diperincikan untuk menjawab persoalan sama ada sukfaktan lipid ester mampu memberi keadaan yang sama pada udang ternak sebelum dijangkiti AHPND.

Kehadiran sel epitelium hepatopankreas di usus udang menunjukkan bukti wujudnya perhubungan kehadiran penyingkiran sel dengan penyakit AHPND. Bukti kehadiran AHPND ini disokong dengan kadar kemandirian yang rendah dan analisis patologi di usus dan tubul hepatopankreas serta kehadiran bakteria *V. parahaemolyticus*. Penyingkiran sel-sel epitelium daripada tubul hepatopankreas secara akut akibat jangkitan bakteria *V. parahaemolyticus* menyebabkan kematian udang di peringkat awal ternakan. Hasil daripada kajian di fasa I dan II mendapati sel-sel epitelium yang tersingkir ini boleh ditemui di usus udang yang mengalami kematian. Adalah dipercayai udang yang telah mengalami fenomena ini akan cuba untuk menyingkirkan sel-sel epitelium ini daripada tubuh badannya sebagai langkah untuk terus hidup. Keadaan ini dilihat boleh memberi satu tempoh untuk pertumbuhan sel yang baru dalam tubul hepatopankres udang tersebut bagi strategi kemandiriannya. Kajian awal ini telah memberi satu idea bagi mengenalpasti status kesihatan udang ternak melalui kehadiran sel-sel epitelium di usus sebagai petunjuk bahawa udang telah dijangkiti bakteria *V. parahaemolyticus*(AHPND) atau agen penyakit lain yang menyerang organ hepatopankreas udang.

Kajian pembangunan kaedah pengesanan kehadiran sel epitelium hepatopankreas di usus udang melalui penilaian skor kad 'SHOS-Spotter' menunjukkan ia boleh digunakan sebagai petunjuk di peringkat ladang untuk mengesan peringkat awal

kerosakan organ hepatopankreas akibat serangan AHPND. Kaedah pengesanan ini dapat membezakan udang yang dijangkiti dengan AHPND melalui kehadiran sel epitelium hepatopankreas di usus bermula dengan rank skor 1 ke atas manakala rank skor 0 menunjukkan udang tersebut tidak mengalami penyingkiran sel epitelium di hepatopankreas atau petunjuk sebagai udang itu tidak mengalami kerosakan organ hepatopankreas atau tidak dijangkiti oleh AHPND. Penilaian kaedah skor kad usus ini membolehkan satu tindakan awal pengurusan dirangka oleh penternak di dalam mengambil tindakan awal mencegah kematian udang. Kaedah mudah dan pantas ini mampu membantu penternak dari mengalami kerugian teruk.

Akhir sekali, keputusan kajian berfasa ini memberi banyak informasi mengenai kejadian penyakit AHPND dan membuka lebih banyak peluang untuk penyelidikan yang lebih terperinci dalam pelbagai aspek bagi menangani masalah yang berkaitan dengan AHPND.

8.0

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8.1 Penerbitan

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8.2 Pembentangan

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3. Kua B.C, Fariddudin M, Ahmad Iftikhar AM, Siti Zahrah A, and Norazila J. *Malaysian Experiences on EMS. Invited speaker,* for Seminar Udang Kebangsaan 2013. 19 March 2013, Kuala Lumpur.

4. Kua B.C, Ahmad Iftikhar AM, Thavallingam M, Chong, F.F, Arif MD & Norazila J. *Status of EMS/AHPNS in Malaysia: Cases and investigation. Invited speaker for FAO/MARD Joint Final Technical & National Consultations on EMS/AHPNS of cultured shrimp. Invited speaker* 25-28 June 2013, Hanoi, Vietnam.

5. Pihak pengurusan Blue Archipelago, Setiu di Terengganu pada 12 Jun 2013

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9. Kua BC., Ahmad Iftikhar AM., Siti Zahrah A., Mohd. Afiq M.,Turni.H & Norazila J. *Early detection of AHPNS /EMS at farm level through the presence of hepatopancreas cells in the gut of cultured Litopenaeus vannamei. Oral presentation*, FRI Konvensyen 2014. 25-26 Feb 2014.

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8.3 Harta Intelek

Satu kaedah pengesanan awal telah dibangunkan berdasarkan penentuan status kesihatan udang melalui pemerhatian sel tertentu dalam usus udang ternakan. Kaedah tersebut dikenali sebagai SHOS-Spotter (Foto 31). Ia telah dibangunkan untuk membantu penternak udang ternak yang sering berdepan dengan isu kematian udang sebelum tindakan diambil. Model SHOS-spotter telah dibangunkan berdasarkan penyakit *Early Mortality Syndrome (EMS)/Acute Hepatopancreas Necrosis Disease (AHPND)* dan parasit mikrosporidian *Enterocytozoon hepatopenaei* (EHP) dalam ternakan udang. SHOS-Spotter boleh mengesan kesihatan udang dalam masa 1-3 jam. Inovasi ini telah didaftar sebagai cap dagang dengan nombor TM2021029905 (*kelas* 10) di bawah Perbadanan Harta Intelek Malaysia (MyIPO) pada tahun 2021 (Rajah 24).

Foto 31: Kit pengesanan kesihatan udang di lapangan yang datang sekali dengan tindakan susulan.

Rajah 24: Cap dagang yang didaftarkan di bawah Perbadanan Harta Intelek Malaysia (MyIPO) pada tahun 2021.

8.4 Anugerah

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9.0

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Detection of *PirA/B* Toxin Genes for Acute Hepatopancreatic Necrosis Disease (AHPND) and *Vibrio parahaemolyticus* in *Penaeus vannamei* Culture from Major White Shrimp Producing Farms in Malaysia

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ABSTRACT

The acute hepatopancreatic necrosis disease (AHPND) epidemic from 2010 to 2013 significantly affected white shrimp (*Penaeus vannamei*) production in Malaysia. This study aims to determine the status of AHPND in *P. vannamei* culture from detecting *PirA/B* toxin genes in hepatopancreas tissues and isolation of *Vibrio parahaemolyticus* for identification of pathogenic strain from major white shrimp producing farms in Malaysia. Bacteria from the hepatopancreas organ were cultured on tryptic soy agar and identified using API® 20 NE (bioMérieux, France) for *Vibrio* species. Confirmation of *PirA/B* toxin genes in hepatopancreas and *V. parahaemolyticus* isolates were determined by polymerase chain reaction (PCR). Twenty-three *V. parahaemolyticus* isolates were identified from 7.7% of the analysed samples. Fourteen (14) (4.7%) were detected with *PirA/B* toxin genes from districts of Johor such as Batu Pahat (1) and Kota Tinggi (8), Alor Setar, Kedah (3), Tawau, Sabah (1), and Kuching, Sarawak (1). In contrast, the other nine isolates (3%) contained only the plasmid. Genomic and phylogenetic tree analysis of four *V. parahaemolyticus* isolates carrying *PirA/B* toxin genes from this study showed that only one strain (Vp14) harbours

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the *PirA/B* complete genes in addition to displaying full sequence homology and coverage to the pVA1 plasmid. In contrast, other strains (AAT22, IKK3, and PK3) displayed partial sequence homology of plasmid harbouring key genes associated with conjugative transfer function but not the plasmid segments containing *PirA/B* toxin genes. Hence, this study showed that six farms were negative from AHPND. In contrast, four farms were positive with *PirA/B* toxin genes in juveniles from Pekan, Pahang (26.7%), Kuching and Sarikei, Sarawak (10% respectively), and Alor Setar, Kedah (3.3%).

Keywords: AHPND, *P. vannamei*, *PirA/B* toxin genes, prevalence, *V. parahaemolyticus*

INTRODUCTION

Acute hepatopancreatic necrosis disease (AHPND) or previously known as early mortality syndrome (EMS), is a bacterial disease caused by a unique strain of *Vibrio* spp., including *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Vibrio owensii*, *Vibrio campbelli*, *Vibrio punensis*, and other possible bacteria that contain ~70-kbp plasmid genes which encode homologous of the *Photorhabdus* insect-related toxins, *PirA/B* (Devadas et al., 2019; Lee et al., 2015; Yang et al., 2014). Nucleotide content [guanine-cytosine (GC)] of these two genes is only 38.2% and is substantially lower than the rest of the plasmid, which suggests that these genes are acquired (Feng et al., 2017; Han et al., 2015). In addition, a plasmid that contains *PirA*/*B* toxin genes was found in the pathogenic AHPND strain of *V. parahaemolyticus* (*VPAHPND*) but was absent in non-pathogenic strain, suggesting *PirA/B* toxin genes as the causative agent for AHPND. These *PirA/B* toxin genes were also found in *Photorhabdus* spp., which are gram-negative, luminescent, rod-shaped bacteria members of the family Enterobacteriaceae. In nature, *Photorhabdus* spp. establish an obligate, symbiotic relationship with entomopathogenic nematode *Heterorhabditis* spp., which are parasites of insect larvae that have a wide geographic distribution. The first detection of AHPND was reported in China in 2009, Malaysia and Vietnam in 2011, Thailand in 2012, and the Philippines in 2015 (Dabu et al., 2015; Food and Agriculture Organization of the United Nations [FAO], 2013, 2016).

Malaysia produced significant quantities of *Penaeus monodon* in the early 2000s but then switched largely to *P. vannamei* until the AHPND epidemic hit the country between 2011 and 2013. Sentinel surveillance based on reports of mortality cases in white shrimp farms from Malaysia showed an increasing number of cases since 2011, with the first report on the east coast of Johor and subsequently in Pahang, Perak, and Penang (Kua et al., 2016). The prevalence of AHPND was 50% and 26% in 2011 and 2012, respectively. Confirmation of AHPND was based on observation of clinical signs and the characteristic pathology of acute and terminal stages of AHPND in hepatopancreas organs (Kua et al., 2016). Pale discolouration and atrophy of hepatopancreas accompanied by loose

shells, empty stomach or discontinuous midgut, and corkscrew swimming behaviour were reported. In addition, mixed bacteria of *Vibrio parahaemolyticus*, *Vibrio fluvialis*, *Vibrio alginolyticus*, *Vibrio cholerae*, *Aeromonas hydrophila*, *Enterobacter cloacae*, *Pseudomonas* spp., and *Photobacterium damselae* were isolated from the hepatopancreas.

About 67% of shrimp production in Malaysia comes from white shrimps (Harkell, 2018). AHPND had caused a significant drop in *P. vannamei* production from 87,000 metric tonnes in 2010 to 67,000 metric tonnes in 2011. A continuous drop in *P. vannamei* production was recorded in 2012 with 48,991.81 metric tonnes (RM61.59 million) to 45, 473.74 metric tonnes (RM86.72 million) in 2013 (Department of Fisheries Malaysia [DOF], 2010, 2011, 2012, 2013). Statistical data in 2018 recorded *P. vannamei* production of 36,007.25 metric tonnes (RM79.8 million) (DOF, 2018). Despite increasing aquaculture areas and shrimp culture farms, diseases and mortalities have been identified as major obstacles to sustainable production. AHPND and hepatopancreatic microsporidians caused by *Enterocytozoon hepatopenaei* (EHP) have been reported as two emerging diseases from 2010 to 2015 that are usually occurred concurrently and have significantly affected shrimp production due to high mortalities (AHPND) and/or stunted growth (EHP) (FAO, 2017).

Giant tiger shrimp (*Penaeus monodon*), white leg shrimp (*Penaeus vannamei*), and oriental/Chinese white shrimp (*Penaeus chinensis*) are known to be infected with AHPND that is characterised by mass mortalities between 40% and 100% in 20–30 days of post-stocking in grow-out ponds (Lightner et al., 2012). Management practices in farms, including pond management and maintenance of good water quality, are known to prevent or avoid stressing shrimp and making them more susceptible to disease. However, low compliance with standards in good biosecurity and good aquaculture practices at farm and hatchery facilities have been identified as major factors favouring the spread of disease from one farm to the other (FAO, 2016). Therefore, shrimp aquaculture needs to continuously develop a systematic approach that implements responsible and science-based farming practices. Hence, the objective of this study is to determine the status of AHPND in *P. vannamei* culture from detection of *PirA/B* toxin genes and bacteria *V. parahaemolyticus* from hepatopancreas organ in relation to several routine management practices of pond culture.

MATERIALS AND METHODS

Sampling Size and Locations

A cross-sectional study on the status of AHPND was carried out from major shrimp producing areas in Malaysia, involving ten shrimp farms from 10 districts in Kedah, Penang, Terengganu, Pahang, Johor, Sarawak, and Sabah that started from January to November in 2019. A minimum of 30 pieces of juvenile shrimps/day of culture (DOC) aged less than 30 or between

31–45 DOC were collected randomly from various pond cultures (maximum 5 to 6 culture ponds/30 pieces). A total of 300 pieces of juveniles were sampled and analysed individually. The sampling sites, states, districts, months of sampling, and the number of samples are shown in Table 1.

Table 1

A sampling of Penaeus vannamei *at the day of culture (DOC) less than 30 days of age, or juveniles between 31–45 days old from 10 locations that show states, districts, months, and number of samples*

States	District	Month (2019)	Number of samples
Kedah	Alor Setar	April	30
Penang	Bkt. Tambun	February	30
Terengganu	Setiu	March	30
Sarawak	Sarikei	May	30
	Kuching	May	30
Johor	Batu Pahat	August	30
	Kota Tinggi	August	30
Pahang	Pekan	October	30
Sabah	Kudat	November	30
	Tawau	November	30
		TOTAL	300

Bacterial Culture, Isolation, and Identification

About 210 pieces of juvenile white shrimps aged less than 30 days old in pond culture (Kedah, Terengganu, Johor, Pahang, and Sabah) and 60 pieces of white shrimps (Sarawak) aged between 30–45 days old were tested to detect the presence of bacteria in the hepatopancreas organ. Bacteria were aseptically inoculated via direct streaking onto tryptic soy agar (TSA), which was incorporated with 1.5% sodium chloride (NaCl) and incubated at 30 °C for 18 to 24 hours, followed by sub-culture until pure isolate was obtained. *Vibrio parahaemolyticus* bacterial was identified using Gram staining, oxidase test, sensitivity to vibrio static 0129-disc agent (2, 4-Diamino-6, 7-di-iso-propylpteridine phosphate) (150/10 µg) and observation of the colour colony on thiosulfate citrate bile salt (TCBS) agar (Austin et al., 1997). A biochemical test for confirmation of *Vibrio* species was carried out using API® 20 NE Kit (bioMérieux, France). The *V. parahaemolyticus* isolates identified with 98% to 99.9% identical to the reference strain in the analytical profile index (API) software database was further tested for the presence of *PirA/B* toxin genes using a PCR

method. An isolate of *V. alginolyticus* was used as rooting for phylogenetic analysis.

DNA Extraction of Hepatopancreas Tissues and Bacteria Cells

A total of 300 hepatopancreas tissues samples in 95% alcohol fixation were tested for *PirA/B* toxin genes using a PCR method. DNA extraction was carried out using the cetyl trimethyl ammonium bromide (CTAB) and dodecyle trimethyl ammonium bromide (DTAB) method (IQ2000™, GeneReach Biotechnology Corp., Taiwan). In contrast, bacteria *V. parahaemolyticus* was extracted using G-Spin™ Genomic Bacteria Extraction Kit (iNtRON Biotechnology, Korea). About 30 mg of hepatopancreas tissues fixed in 95% alcohol was placed into a 1.5 mL tube containing 600 µL DTAB solution. Tissues were ground with a sterile disposable grinder until they were completely dissolved into DTAB solution. After that, they were incubated at 75 °C for 5 minutes and cooled down to room temperature. The mixture was vortexed and spun down briefly and was then added with 700 µL of chloroform and centrifuged at 12,000 x *g* for 5 minutes. Next, the upper aqueous phase was transferred into a new 1.5 mL tube and added 100 µL of CTAB solution and 900 µL of deionised water. It was then vortexed briefly, incubated at 75 °C for 5 minutes and centrifuged at 12,000 x *g* for 5 minutes. Next, the pellet was re-suspended with 150 µL dissolving solution, incubated at 75 °C for 5 minutes and spun at 12,000 x *g* for 5 minutes. Finally, the supernatant was transferred into a new 1.5 mL tube containing 300 µL of 95% ethanol. This procedure was repeated twice, whereby the pellet was washed with 75% ethanol in the last procedure. The final pellet was dried and dissolved in deionised water or Tris ethylenediaminetetraacetic acid (TE) buffer. Dissolving DNA was stored at -20 °C until used for polymerase chain reaction (PCR).

Total genomic DNA from *V. parahaemolyticus* isolate was extracted using G-spin™ Kit (iNtRON Biotechnology, Korea). About 1 mL of bacteria cells was harvested from an overnight culture (18–24 hours) at 30 °C in tryptic soy broth incorporated with 1.5% NaCl (OD_{600} 0.8–1.0) by centrifugation at 13,000 x *g* for 1 minute. The supernatant was removed, and cells were re-suspended by vortex and tapping. Bacteria cells pellet was extracted according to the manufacturer's instructions. The final collected DNA in a 1.5 mL tube was measured using a NanoDrop™ spectrophotometer (DS-11 Series, DeNovix, USA).

PCR Reaction Conditions

Nested PCR using AP4 primers was followed with some optimisation of annealing temperature using Maxime PreMix *i-Taq* (iNtRON Biotechnology, Korea) for detecting *PirA/B* toxin genes at 230 bp portion of a sequence, which includes 209 bp of the *ToxA* or *PirA* gene sequence plus 12 bp spacer sequence plus 9 bp of succeeding *ToxB* or *PirB* gene sequence (Dangtip et al., 2015). First-step PCR was performed using primer AP4-F1 with the sequence: 5'-ATGAGTAACAATATAAAACAT

GAAAC-3' and AP4-R1: 5'-ACGATTTCGACGTTCCCCAA-3', followed by nested PCR using AP4-F2 primer: 5'-TTGAGAATACGGGACGTGGG-3' and AP4-R2: 5'-GTTAGTCATGTGAGCACCTTC-3'. In the first PCR reaction, 20 µL of the total volume reaction mixture was prepared, which consisted of DNA template 2 µL, AP4-F1 primer (0.4 µL 100 pmol/µL), AP-R1 primer (0.4 µL 100 pmol/µL), and deionised water (17.2 µL). Then, amplification was performed with the following parameters: initiation denaturation at 94 °C for 2 minutes, followed by 35 cycles of 94 °C for 30 seconds, 53 °C for 30 seconds, and 72 °C for 90 seconds, and a final extension at 72 °C for 2 minutes. For the second step (nested) PCR reaction, 1 µL of the final solution from the first-step PCR was diluted with deionised water at the ratio of 1 to 100, 2 µL of the diluted solution was used with nested primers AP4-F2 (0.4 µL 100 pmol/µL), AP4-R2 (0.4 µL 100 pmol/µL), and deionised water (17.2 µL) were used to make up for 20 µL of total volume. Then, amplification was performed with initiation denaturation at 94 °C for 2 minutes, followed by 25 cycles of 94 °C for 30 seconds, 53 °C for 30 seconds, 72 °C for 30 seconds, and a final extension of 72 °C for 2 minutes. Following PCR, an aliquot of PCR products was analysed in a 1.5% gel containing green fluorescent dye nucleic acid staining solution (RedSafe™, iNtRON Biotechnology, Korea).

Sample Preparation for Sequencing and Phylogenomic Tree

Genomic deoxyribonucleic acid (gDNA) of five *Vibrio* spp., identified as IKK3, AAT22, PK3, Vp14, and SK6, were sent for sequencing via the iSeq100 Next Generation Sequencing System (GeneSEQ, Malaysia). Genome completeness analysis via BUSCO v4 was assessed for each assembled genome. In addition, protein-coding genes were briefly predicted from the assembly and were assessed for the presence of 1445 conserved genes found in *Vibrio* (vibrio_odb10). As expected from the high-quality assembly, each genome assembly exhibited genome completeness of >99.9%. The gDNA was fragmented using a Covaris ultra sonicator to approximately 350 bp. The fragmented DNA was end-repaired, adapter-ligated, and PCR-enriched using the NEBNext® Ultra™ II DNA Library Preparation Kit (New England Biolabs, USA) according to the manufacturer's instructions. The constructed libraries were normalised and sequenced on NovaSeq 6000 System (Illumina, USA) using a 2 x 150 bp read configuration (Simão et al., 2015).

Vibrio parahaemolyticus genome assemblies in National Center for Biotechnology Information (NCBI) that originated from Malaysia were used to infer a phylogenomic tree of five isolates (IKK3, AAT22, PK3, Vp14, and SK6) obtained from this study. The complete genome sequence of *V. parahaemolyticus* strain MVP1 was used as the reference genome. Each genome was subsequently aligned to

this reference genome to identify single nucleotide polymorphisms (SNPs) and generate a core SNP alignment. The Snippy v4.6.0 pipeline was used to perform these tasks. First, a maximum-likelihood tree was constructed using the FastTree2 setting, followed by visualisation and annotation in Figtree v1.4.1 (Price et al., 2010).

RESULTS

Biochemical Confirmation of Bacteria Isolates

Twenty-three isolates of *Vibrio* spp. were subjected to a biochemical test to confirm the species using API® 20 NE (bioMérieux, France). Isolates Vp14, IKK3, AAT22, and PK3 were identified as *V. parahaemolyticus* with 90% to 99.1% identical to the reference strain in the API system. They were Gram-negative halophilic bacteria, which produced green colony growth on thiosulfate citrate bile salt sucrose (TCBS) agar, was sensitive to vibrio static 0129-disc agent (150/10 µg) and exhibited cytochrome oxidase with catalase activity. Enzymatic assays showed that they were nitrate (NO_3) reductase, tryptophanase, glucose fermentation, gelatinase, and β=galactosidase but produced a negative reaction to arginine dihydrolase, urease, and esculin hydrolase. Carbohydrate assimilation showed a positive reaction to D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, maltose, D-gluconate, and L-malate but a negative reaction to caprate, adipate, citrate, and phenylacetate. *Vibrio alginolyticus* (SK6) produced similar results as *V. parahaemolyticus* isolates, except that it did not produce acid from glucose or decarboxylate β-galactosidase and did not break up arabinose.

Prevalence of AHPND from Detection of *PirA/B* Toxin Genes in Hepatopancreas of *Penaeus vannamei*

The highest prevalence of AHPND was found in juveniles/day of culture (DOC) of white shrimps from Pekan (8, 26.7%), Pahang. In contrast, the low prevalence was recorded in juvenile shrimps from Sarikei (3, 10%), Kuching (3, 10%), Sarawak, and Alor Setar (1, 3.3%), Kedah. The 7.3% of samples (22) were tested positive with *PirA/B* toxin genes with an overall mean prevalence of 5%. Results are shown in Table 2. AHPND prevalence was diagnosed by detecting *PirA/B* toxin genes from hepatopancreas tissues of white shrimps fixed in 95% alcohol and supported with or without the isolation of *V. parahaemolyticus* isolate that carries *PirA/B* toxin genes. AHPND was not detected in hepatopancreas tissues of juvenile white shrimps from Johor (60), Sabah (60), Terengganu (30), and Penang (30). However, culture isolates of *V. parahaemolyticus* obtained from white shrimp hepatopancreas at Kota Tinggi (8, 26.7%) and Batu Pahat (1, 3%) showed that these strains are pathogenic which carry *PirA/B* toxin genes.

Table 2

Detection of AHPND with PirA/B *toxin genes from the hepatopancreas of juvenile/DOC white shrimps*

States	District	Sample number (n)	Positive sample for *PirA/B* toxin genes (Prevalence, %)
Kedah	Alor Setar	30	1(3.3)
Penang	Bukit Tambun	30	-
Terengganu	Setiu	30	-
Johor	Batu Pahat	30	-
	Kota Tinggi	30	-
Pahang	Pekan	30	8(26.7)
Sarawak	Kuching	30	3(10)
	Sarikei	30	3(10)
Sabah	Kudat	30	-
	Tawau	30	-
Total		300	15(50)
Mean (%)			5.0

Note. Mean (%): (Number of the sample with *PirA/B* toxin genes/Total samples) x 100%

Prevalence of *Vibrio parahaemolyticus* with *PirA/B* Toxin Genes

Twenty-three (23) *V. parahaemolyticus* isolates were obtained from 270 samples of juvenile white shrimps, whereby 14 isolates were detected with *PirA/B* toxin genes. AHPND isolates were found in samples from Kota Tinggi (8, 26.7%) and Batu Pahat (1, 3.3%), Johor, Alor Setar, Kedah (3, 10%), and Kudat, Sabah (1, 3%). Although *V. parahaemolyticus* bacteria from culture media isolation were detected to have *PirA/B* toxin genes, direct PCR analysis of hepatopancreas tissues fixation (95% alcohol) of the similar samples failed to detect *PirA/B* toxin genes from these tissue samples. Virulence of AHPND-causing *V. parahaemolyticus* depends on the amount of *PirA/B* toxin released and caused cellular damage to hepatopancreas when *V. parahaemolyticus* bacteria initially colonise in the shrimp stomach and eventually reach the hepatopancreas (Han et al., 2015). In this situation, *V. parahaemolyticus* was considered a relatively non-virulent bacterium until it released a potent toxin (*PirA/B*) in the host tissues/hepatopancreas organ or induced the clinical disease condition and mortality in white shrimps. PCR analysis of hepatopancreas tissue of white shrimp sample was negative from Johor (60) and Sabah (60). The detection of *PirA/B* toxin genes from *V. parahaemolyticus* isolates obtained through the propagation of bacteria cells in the laboratory was not considered a positive case

for AHPND in this surveillance study but rather for identifying the pathogenic strain. PCR analysis of the cultured cells showed that 13 *V. parahaemolyticus* AHPND strains were identified from Kota Tinggi (8) and Batu Pahat (1), Johor; Alor Setar (3), Kedah, and Kudat (1), Sabah. Another study has shown that infection of AHPND depends on sufficient bacteria cells count to secrete or release *PirA/B* toxins in the shrimp tissues rather than the number of copies of toxin genes (Tinwongger et al., 2016).

Genomic Sequence of *PirA/B* Toxin Genes and pVA1 Plasmid

Five isolates of *V. parahaemolyticus* from Sarawak (Vp14), Kedah (IKK3), Johor (PK3, AAT22), and *V. alginolyticus* from Sabah (SK6) were subjected to whole-genomic sequences to determine the virulence of local strains obtained from this study. The whole-genome sequences were aligned to the pVA1 plasmid using blastN with an E-value of $1e^{-50}$ and subsequently visualised in Blast Ring Image Generator (Alikhan et al., 2011; Dong et al., 2019). Being consistent with the initial PCR screening result, strain Vp14 (Sarawak) was the only strain that harboured the *PirA/B* complete genes in addition to displaying full sequence homology and coverage to the pVA1 plasmid. In contrast, strain AAT22 (Johor) displayed significant sequence homology with at least 50% length of the plasmid harbouring key genes associated with conjugative transfer function but not the plasmid segment containing the *PirA/B* genes (Figure 1).

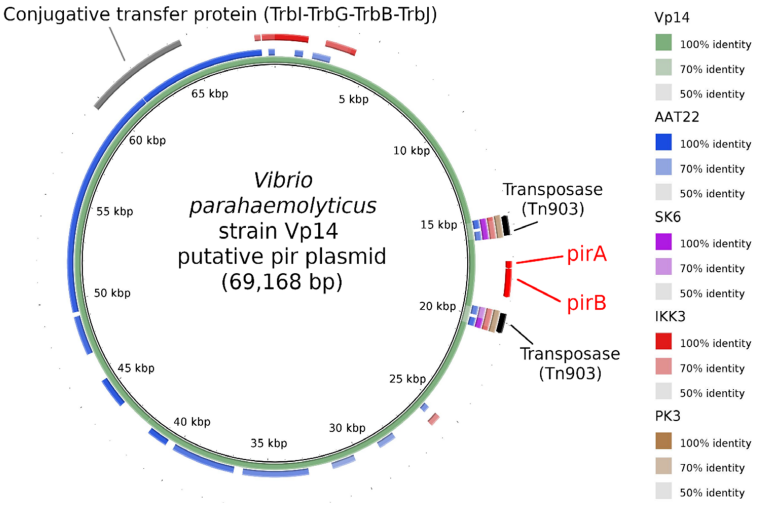

Figure 1. Circular visualization of the pVA1 plasmid and selected genes. Coloured rings indicate a genomic region with significant homology to the strain of interest (Vp14, AAT22, SK6, IKK3, and PK3)

Phylogenomic Tree of *Vibrio parahaemolyticus* Associated with Genome Assemblies in NCBI from Aquaculture in Malaysia

Four *V. parahaemolyticus* isolates, which were identified as Vp14 (Sarawak), AAT22 and PK3 (Johor), IKK 3 (Kedah), and SK6 (Sabah), were identified as *V. alginolyticus* that were subjected to genomic and phylogenetic analysis. The assembled genomes were compared to *Vibrio* spp. type strains to confirm their taxonomic assignment. Strains AAT22, IKK3, PK3, and Vp14, showed a pairwise average nucleotide identity of more than 98% to *V. parahaemolyticus*. In contrast, strain SK6 showed an average nucleotide identity of 98.5% to *V. alginolyticus* and less than 90% to other tested *Vibrio* species. Strain SK6 (Sabah) expectedly displayed a long branch length and was thus chosen as the outgroup for rooting, given its taxonomic assignment as *V. alginolyticus*. Strain Vp14 harbours the *PirA/B* toxin genes formed a monophyletic cluster with high SH-like support of a few MVP strains isolated from a shrimp pond water sample located in Negeri Sembilan in 2016. In contrast, strains IKK3 (Kedah), AAT22 and PK3 (Johor) only shared a relative distance ancestor with some of the publicly available strains, as evidenced by their relatively longer branch length (Figure 2). It suggested that they could be novel genomic lineages of *V. parahaemolyticus,* which was not reported previously in Malaysia.

DISCUSSION

This study showed that grow-out or juvenile white shrimps aged less than 30 days of culture in pond and post-larvae appeared most susceptible to AHPND infection with an overall mean prevalence of 5% and 4.7%, respectively. AHPND often occurs within 20–30 days of post stocking in grow-out ponds and causes mass mortalities in post-larvae shrimps (De Schryver et al., 2014). Therefore, most farms practise routine screening for major shrimp diseases that are known in aquacultures, such as white spot disease that is caused by white spot syndrome virus (WSD/WSSV), yellow head virus (YHV), taura syndrome virus (TSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV), AHPND, and EHP as parts of prevention programmes in their farms whereby the infected stocks will be destroyed.

This study identified 23 *V. parahaemolyticus* isolates from 7.7% of total samples. Fourteen (14) strains of *V. parahaemolyticus* were found to have *PirA/B* toxin genes, whereas nine isolates were detected with plasmid. Gross observation during sampling showed only mild changes in hepatopancreas organs with pale coloured, atrophied, empty midgut, and soft shell in several samples, suggesting that subclinical infection may go unnoticed. However, AHPND infection caused by *V. parahaemolyticus* that produce *PirA/B* toxin genes will usually cause severe pathological changes to hepatopancreas organ, which are shown via degeneration and massive sloughing of hepatopancreas cells, followed

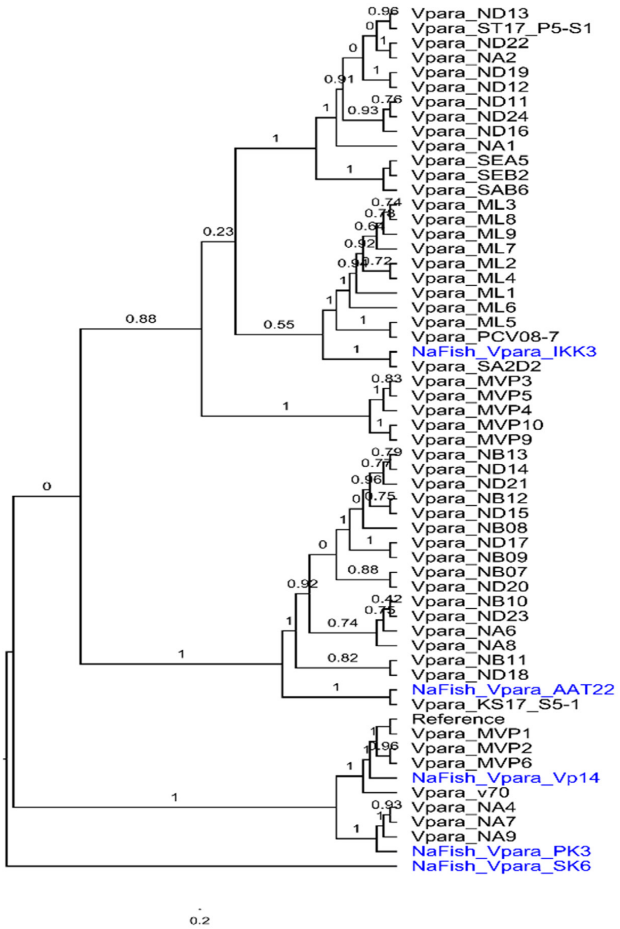

Figure 2. Maximum likelihood tree constructed based on the core SNP alignment of publicly available Malaysian *Vibrio parahaemolyticus* genomes and NaFish *Vibrio* genomes (blue labels). Strain SK6 was rooted as the outgroup, given its taxonomic classification as *Vibrio alginolyticus*. The branch lengths indicate the number of substitutions per site, while the SH-like bootstrap supports values that were indicated by the colour of the nodes.

by high mortalities in disease outbreaks (Lightner et al., 2012; Nunan et al., 2014). However, typical gross pathological changes associated with clinical disease of AHPND was not observed on-site, and farmers have not reported high mortalities. Nevertheless, the occurrence of *V. parahaemolyticus* bacteria with *PirA/B* toxin genes in the hepatopancreas of shrimp will increase their risk to AHPND under stressful conditions. Routine management practices applied in farms such as regular health screening of stocks, strict biosecurity measures, hygienic practices at the entrance and within farms culture area, and pond management and its water quality are among many factors that impact the management of disease-free culture.

The pVA1 plasmid is the source of the AHPND-causing toxin, whereas *PirA/B genes*, irrespective of other plasmidic factors of pVA1, are sufficient to produce symptoms associated with AHPND (Lee et al., 2015). Culturable cells of *V. parahaemolyticus* local strains were recovered from -20 ^{o}C storage. Genomic sequencing of four local strains of *V. parahaemolyticus* carrying *PirA/B* toxin genes showed that only one strain (Vp14) harboured the *PirA/B* complete genes, which displayed a full sequence of pVA1 virulent plasmid. The risk of AHPND outbreak can be reduced by controlling the *Vibrio* spp. activities, in particular, *V. parahaemolyticus* cells count. The bacterial concentration ranging ($5 \times 10^4 – 5 \times 10^5$ cfu/ml) from AHPND strain has been proven to be able to cause significant mortalities to *P. vannamei* from 60% to 100% within 3 to 6 days of post infections from the immersion challenge test (Tinwongger et al., 2016). Hence, the virulence of AHPND depends on sufficient bacterial count to release or secrete toxin rather than the number of copies of toxin genes (Tinwongger et al., 2016). Other studies showed that phytoplankton/green water and nutrient enrichment affect the microbial community in the ecosystem, especially bacterial load and interaction with the shrimp immune responses (De Schryver et al., 2014). Probiotic and/or algae-rich green waters are known to be able to create microbially matured water systems, whereby environments that are primarily colonised by slow-growing harmless bacteria may best guarantee the prevention of AHPND outbreaks (De Schryver et al., 2014). Probiotics and molasses help to increase the diversity of heterotrophic bacteria, including *V. parahaemolyticus* thus, effectively inhibiting pathogens (Bhatnagar & Pooja, 2013; Hu et al., 2016). Molasses are known to improve water quality, and it is suggested to be used during the nursery and grow-out phase of *P. vannamei* under limited water discharge or close system (Tzachi et al., 2007).

CONCLUSION

The health status of AHPND from grow-out/juvenile white shrimps aged less than 30 days of culture was determined through a cross-sectional study using a random sampling method. AHPND with the detection of *PirA/B* toxin genes and identification of *V. parahaemolyticus* with *PirA/B* toxin genes were determined from 10 major shrimp

producing areas in Malaysia. AHPND with *PirA/B* toxin genes detection from the hepatopancreas of white shrimps was found in Kuching and Sarikei, Sarawak at 10% prevalence, respectively, followed by 3.3% in Alor Setar, Kedah, and 26.7% from Pekan, Pahang. *PirA/B* toxin genes of AHPND were not detected in white shrimps from farms in Penang, Johor, Terengganu, and Sabah. Genomic and phylogenetic tree analysis of four *V. parahaemolyticus* isolates carrying *PirA/B* toxin genes from this study showed that only one strain (Vp14) harboured the *PirA/B* complete genes in addition to displaying full sequence homology and coverage to the pVA1 plasmid. In contrast, other strains (AAT22, IKK3, and PK3) displayed partial sequence homology of plasmid harbouring key genes associated with conjugative transfer function but not the plasmid segments containing the *PirA/B* toxin genes.

Many factors are known to influence the clinical manifestation of AHPND, such as the presence of *PirA/B* toxin genes and *V. parahaemolyticus* bacteria that carry *PirA/B* toxin genes in hepatopancreas tissues, as well as water quality and bacterial cell counts in pond culture. Health screening through regular observation of shrimp behaviour and health checks for important diseases in shrimp culture provides the best solution for early detection and management of health problems. The risk of spreading the disease to other farms can be prevented through removal and safe disposal of sick or dead shrimps, as well as emergency harvesting if necessary, or destruction of infected stocks as appropriate. Strict biosecurity measures and disinfection have been strictly implemented in almost all farms that the researchers visited or surveyed. This practice is believed to contribute significantly to controlling and preventing AHPND.

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Mortality Outbreaks in Whiteleg Shrimp (*Penaeus vannamei* Boone 1931) Cultured in Peninsular Malaysia

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Abstract

The whiteleg shrimp (*Penaeus vannamei* Boone 1931) was introduced for farming in Malaysia in early 2002. In 2009, reports of early mortality syndrome (EMS) were noted in the People's Republic of China and Viet Nam. One form of EMS, acute hepatopancreatic necrosis disease (AHPND), has now spread to several shrimp-growing countries in Asia. In 2011, Malaysia recorded a mortality outbreak that prompted an investigation of 20 farms where 204 moribund shrimp samples were analyzed. On average, 64 % of the affected shrimp showed haemolymph clotting time longer than 1.5 min, and 80 % had pale hepatopancreas, soft body and empty gut. Multiple bacterial infections, particularly *Vibrio* spp. and *Photobacterium damsela,* were isolated from the haemolymph and hepatopancreas of affected shrimp. *Vibrio parahaemolyticus* was detected positive for the *tox*R gene.

Histopathology showed massive sloughing of the epithelial cells of the hepatopancreatic tubules and multifocal septic and melanized hepatopancreatic tubules that were encapsulated by haemocytes. Tests by polymerase chain reaction (PCR) were negative for infectious myonecrosis virus (IMNV) (0/110) and only a low prevalence (7/196) of infectious hypodermal and haematopoietic necrosis virus (IHHNV) was recorded. Infected shrimp also tested positive for paralytic shellfish poison (PSP) (24/24), and rearing water samples showed ammonium, nitrate, sulfide and iron levels above the optimal range for culture purposes.

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In 2012, samples were detected positive for EMS/AHPND using the IQ2000 Ems2 detection kit. The findings from this investigation showed that shrimp had multiple bacterial infections and pathological changes consistent with AHPND; some affected shrimp were positive for IHHNV and PSP toxin. These findings support the conclusion that mortalities were due to EMS/AHPND.

Keywords: acute hepatopancreatic necrosis disease, bacteria, early mortality syndrome, Malaysia, *Penaeus vannamei,* shrimp

Introduction

The shrimp culture industry in Malaysia has inevitably suffered major epizootics due to viral infections. In 1996, white-spot syndrome virus (WSSV) affected many farms culturing giant tiger prawn (*Penaeus monodon* Fabricius 1798) in northern Peninsular Malaysia, crippling the industry. The operators were forced to shift to fish culture or, in some cases, cease operation. In 1999, specific pathogen free (SPF) whiteleg shrimp (*P. vannamei* Boone 1931) were introduced, and this encouraged most farmers in Southeast Asia to abandon giant tiger prawn culture in favour of whiteleg shrimp. Malaysia was no different, and whiteleg shrimp was introduced in early 2002 (Briggs et al. 2004). After a few cycles of cultivating whiteleg shrimp, production was reported to have increased significantly; the production of whiteleg shrimp in 2005 was 11 497 tonnes, which increased to 18 601 tonnes in 2006, exceeding that of giant tiger prawn (DOF 2005, 2006). In 2010, total production of whiteleg shrimp reached 69 084 tonnes, 50-fold more than giant tiger prawn, indicating that whiteleg shrimp is a better species to culture (DOF 2010). *Penaeus vannamei* was seen to have faster growth rate, was perceived to have better tolerance to ammonia and nitrite toxicity, and showed higher survival. Generally, three to four crops a year could be produced, as each crop requires only 80–90 days.

Penaeus vannamei is non-indigenous to Asia, and concern about negative impacts such as the introduction of Taura syndrome virus (TSV), infectious myonecrosis virus (IMNV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV) accompanied its introduction. TSV, IMNV and IHHNV are diseases listed by the World Organisation for Animal Health (OIE). TSV and IMNV have caused high mortalities compared to IHHNV (Lightner 1996) and may pose a risk to culture sites and the local shrimp industry. Following the availability of SPF *P. vannamei* postlarvae (PL) and the ability to adapt to a wide range of salinity (0.5 to 28 ppt), culture of whiteleg shrimp spread rapidly (Pan et al. 2007). Unfortunately, both IMNV and TSV have been detected and have caused mass mortality in cultivated *P. vannamei* in Indonesia (Taukhid and Nur'aini 2009). In 2006, Indonesia was the first country in the Asia-Pacific to report mass mortality of *P. vannamei* because of IMNV, the gross signs of which include white necrotic areas or reddening in the muscle of the distal abdominal segments and the tail fan.

After viral disease outbreaks, farms in Asia observed rapid mortalities of *P. vannamei* in the first 30 days of culture. Initially called early mortality syndrome (EMS), affected shrimp were lethargic, anorexic and showed severe damage in the hepatopancreas (Lightner et al. 2012). EMS was first detected in cultivated shrimp in the southern part of the People`s Republic of China in 2009. Slow mortality occurred during the early days of culturing (20–30 days after stocking), and mortality could reach 100 %. In April 2011, farms in Viet Nam experienced 65 to 90 % mortality of *P. vannamei* during the first 45 to 50 days following stocking. Similar scenarios were seen in Thailand in 2012, but mortalities there started at 15 days post-stocking and lasted until 40 days of culture. Sirikharin et al. (2015) reported that a unique strain of the bacterium *Vibrio parahaemolyticus* capable of producing soluble toxins has been identified as the causative agent of acute hepatopancreatic necrosis disease (AHPND). Histopathological observations revealed massive sloughing of the epithelial cells of the hepatopancreatic tupules as a result of the toxins released by this unique bacterial strain.

In 2011, farms culturing *P. vannamei* in the Malaysian states of Perak, Pahang and Penang reported problems similar to AHPND. During investigation, it was found that there were multiple bacterial infections which included *V. parahaemolyticus*. In addition, massive sloughing of the epithelial cells of the hepatopancreatic tubules was also seen in Perak, where 60 % mortality was reported in *P. vannamei* at 20 days of culture (DOC). The remaining stock survived for 50 days, but mortality had reached 90 % by then. In Pahang and Penang, slow mortality was observed at 30–60 DOC. The number of samples from shrimp disease outbreaks submitted to the National Fish Health Research Division (NaFisH) in Penang has been increasing since 2011. Hence, investigations were carried in the states of Perak, Pahang and Penang, northern Malaysia, to confirm the cause of mortalities and determine the factors associated with these outbreaks.[1]

Materials and Methods

Sources of Penaeus vannamei *and Gross Observations*

Two investigations (Phases I and II), with 2–3 months duration each were carried out at different periods in late 2011 and early 2012 (Table 1).

[1] During the period covered by this investigation (2011–2013), we confirmed disease outbreak due to AHPND based on our histological findings (massive sloughing of the hepatopancreatic tubule epithelial cells) and the presence of *V. parahaemolyticus*. Subsequently, in 2014, the samples were confirmed positive using the IQ 2000 ems 2 kit.

Table 1. Investigations carried out during the study period.

Phase	Period	Type of investigation
1	Nov – Dec 2011	Case history
		Gross observation
		Haemolymph clotting time
		Bacteriology
		Histopathology
2	Jan – Mar 2012	Virology (infectious myonecrosis virus (IMNV), Penaeus vannamei nodavirus (PvNv) & infectious hypodermal and haematopoietic necrosis virus (IHHNV)
		Cross-sectional study on the chemical parameters of water quality with special reference to day of culture (DOC) and un-ionized ammonia (NH_3)
		Detection of paralytic shellfish poison (PSP) by enzyme-linked immunosorbent assay (ELISA)

Table 2. Sample information collected from Perak, Pahang and Penang.

Location	DOC upon sampling[1]	Mortality period (DOC)	Source of PL/health status	Survival rate (%)	Total production (tonnes)
Perak					
Farm 1	40	27 & 40	Hatchery 1/unknown	7.0	1.7
	43	30	Hatchery 2/unknown	37.0	4.4
Farm 2	47	20 & 47	Hatchery 3/SPF	10.0	0.4
	48	20	Hatchery 3/SPF	49.0	3.9
Farm 3	40	unknown	Hatchery 4/SPF	36.0	6.6
Farm 4	33	30	Hatchery 5/unknown	29.0	4.4
Pahang					
Farm 1	52	unknown	Hatchery 4/SPF	44.0	3.0
	56	unknown	Hatchery 4/SPF	44.0	3.0
	56	unknown	Hatchery 4/SPF	27.0	3.0
Farm 2	65	45	Hatchery 3/SPF	83.0	8.0
	48	unknown	Hatchery 3/SPF	25.0	2.0
	54	36	Hatchery 4/SPF	14.0	0.5
	52	31	Hatchery 4/SPF	34.0	2.0
Farm 3	84	40	Hatchery 4/SPF	94.0	6.0
	85	40	Hatchery 4/SPF	33.0	2.0
	89	40	Hatchery 4/SPF	24.0	2.0
	89	40	Hatchery 4/SPF	27.0	3.0
Penang					
Hatchery 1	unknown	unknown	Penang	unknown	unknown
Farm 1	50	48	Hatchery 1	unknown	unknown
	50	48	Hatchery 1	unknown	unknown

[1]Abbreviations: DOC = day of culture, PL = postlarvae, SPF = specific pathogen free.

A total of 204 moribund *P. vannamei* samples from 20 farms were collected from the three states in northern Malaysia and processed for haemolymph clotting time, bacteriology, virology and histopathology after gross observations were recorded (Table 2; Fig. 1).

Fig. 1. Map of Peninsular Malaysia showing the three states (*) from which samples were collected during the current study.

Water Quality

The physical parameters of the pond water (i.e. temperature, pH, salinity and dissolved oxygen) were taken *in situ* using a YSI portable meter, while the chemical parameters (i.e. ammonium, nitrate, sulfide and iron content) were analyzed in the laboratory by transporting the samples in a cool box with ice. Ammonium and nitrite were measured using Nessler and diazotization methods, respectively. Other chemical parameters were determined by using reagent kits and read by the Hach spectrophotometer 8038.

Haemolymph Clotting Time

Approximately 0.1–0.2 mL of haemolymph from each shrimp was withdrawn using a 1 mL sterile syringe. Samples were immediately dispensed in drops on a clean slide for observation of haemolymph clotting time. A clotting time of between 1 and 1.5 min was considered normal, while a clotting time higher than 1.5 min was considered as abnormal. Five to ten shrimp from each pond were tested.

Bacteriology

A drop of shrimp haemolymph was inoculated on trypticase soy agar (TSA) plates (Oxoid Ltd., England) and dominant bacterial colonies were subcultured on TSA to obtain pure bacterial isolates. Gram-staining of purified isolates was done and Gram-negative bacteria were subjected to presumptive classification test using *Vibrio* selective medium (TCBS, Merck, Germany), oxidation-fermentation test (OF), vibriostat 0/129 reaction (Oxoid Ltd., England), oxidase reaction using detection paper (Premier Diagnostics Ltd., Malaysia) and motility. Identification of isolates was done using the API 20E and 20NE (BioMerieux, France) identification strips, and bacterial profiles were determined using APIWEB software (BioMerieux, France) and the methods described by Holt et al. (1993). For identification of Gram-positive bacteria, pure cultures of were subjected to a catalase enzyme test using H_2O_2 as a substrate to differentiate between the *Staphylococcus* and the *Streptococcus* group. API 20 STAPH and API 20 STREP identification strips (BioMerieux, France) were inoculated, and bacterial profile was similarly determined by APIWEB software and the methods described by Holt et al. (1993).

Histopathology

Histology was done following Bell and Lightner (1988). A total of 85 live juvenile and adult *P. vannamei* were injected with Davidson's fixative and processed by an automatic tissue processor (Leica ASP 300) following standard procedures. Paraffin sections were affixed on slides and stained with haematoxylin and eosin (H&E).

Molecular Biology

The samples were tested for possible association with viruses known to infect *P. vannamei* in farms. Detection of IHHNV and EMS/AHPND infections was conducted by polymerase chain reaction (PCR) using the IQ2000 kit protocol, while testing for the presence of IMNV and PvVN was performed using the IQ Plus and IQ Real quantitative system distributed by Farming IntelliGene Tech. Corp., respectively. The samples were also tested for *toxR* gene according to Kim et al. (1999).

Cross-Sectional Study on the Chemical Parameters of Water Quality with Special Reference to Culture Period and Un-ionized Ammonia (NH_3)

Five farms in Perak State with different culture periods were selected for a cross-sectional study. Water samples were taken from the ponds and transported inside a cooler box with ice to the laboratory. The parameters that were investigated during the study were ammonium, nitrite, nitrate, sulfide and iron.

Statistical Analysis

The data for physical and chemical parameters were analyzed using One-way ANOVA (SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.). When significant differences were found, the Tukey method for multiple comparisons of means was applied to identify the differences between parameters ($P < 0.05$).

Results

Mortality was observed in all farms in the states of Perak, Pahang and Penang that were visited and where shrimp samples were taken. Gross signs observed in surviving shrimp during sampling included black gill, white faeces, black spot/patches on the exoskeleton, white muscle, white tail/body, reddish body, soft body, yellow discolouration in the head, enlarged hepatopancreas and swimming at pond edges, as well as slow growth.

Shrimp Samples from Perak State

Gross observation of affected shrimp showed signs of poor feeding, swimming at pond edges, white faeces, black spot or patches on the exoskeleton, yellowish head, slow growth, white body/tail and reddish body. However, no black gills were observed among the affected shrimp from four of the farms. Approximately 90–100 % of the examined shrimp showed whitish patches in the abdominal segments, and 80 % had pale hepatopancreas, soft body and empty gut. All ponds had dead shrimp at 20 and 40–50 DOC except for Farm 3 from Sg. Limau, Perak. Seventeen of 20 tested shrimp showed haemolymph clotting time longer than 1.5 min, indicating that 85 % of the tested shrimp were under stress (Table 3).

Table 3. Percentage of haemolymph clotting time tested in affected *Penaeus vannamei* obtained from Perak, Pahang and Penang.

Location	Number of shrimp tested	No. shrimp with haemolymph clotting time exceeding 1.5 min	Percentage of stressed shrimp
Perak	20	17	85
Pahang	30	15	50
Penang	22	14	63

Vibrio spp. and *Photobacterium damsela* were isolated from the haemolymph of the affected shrimp samples from three of the four sites in Perak. Early and terminal phases of EMS pathology were observed in the hepatopancreas and muscle of shrimp from all three sites (Table 4).

The proximal part of the hepatopancreas lacked B, F and R cells, and showed sloughing and necrosis of hepatopancreatic cells (Fig. 2). Multifocal septic and melanized hepatopancreatic tubules with haemocyte encapsulation were also seen in some of the specimens (Fig. 3A). Focal acute necrosis with no obvious agent associated with the lesions was seen in the muscle (Fig. 3B). Water quality parameters such as temperature, pH, salinity and dissolved oxygen were within acceptable ranges for marine shrimp culture (Table 5). However, ammonium, nitrate, sulfide and iron exceeded the normal range recommended for shrimp culture. A cross-sectional study on different days of culture from one farm and five farms showed high ammonium levels on a different DOC (Fig. 4; Table 6). A similar scenario of high levels of ammonium and nitrite also occurred at less than 25 DOC (Fig. 5).

Table 4. Diagnostic results in affected *Penaeus vannamei* obtained from Perak, Pahang and Penang.

Location	Bacteriology	Molecular biology (PCR)[1]	Pathology (%)
Perak	*Vibrio* spp.		Early & terminal stage of EMS (100)
	Vibrio parahaemolyticus	7/64 +ve IHHNV	
		3/3 +ve *tox*R	
	Photobacterium damselae	3/3 +ve EMS/AHPND	
Pahang	*Vibrio* spp.	0/110 +ve IHHNV, PvNv & IMNV	Early & terminal stage of EMS (100)
Penang	Nil	0/22 +ve IHHNV	Early & terminal stage of EMS (100)

[1]Abbreviations: AHPND = acute hepatopancreatic necrosis disease, EMS = early mortality syndrome, IHHNV = infectious hypodermal and haematopoietic necrosis virus, IMNV = infectious myonecrosis virus, PCR = polymerase chain reaction, *tox*R = toxin operon gene.

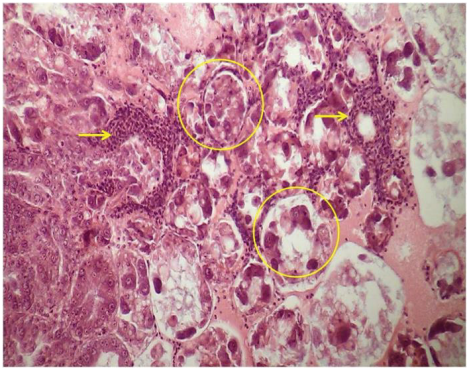


Fig. 2. Proximal hepatopancreas with no B, F or R cells, sloughing (in circle) and necrosis of hepatopancreatic cells with some haemocyte-encapsulated necrotic tubules (arrows).

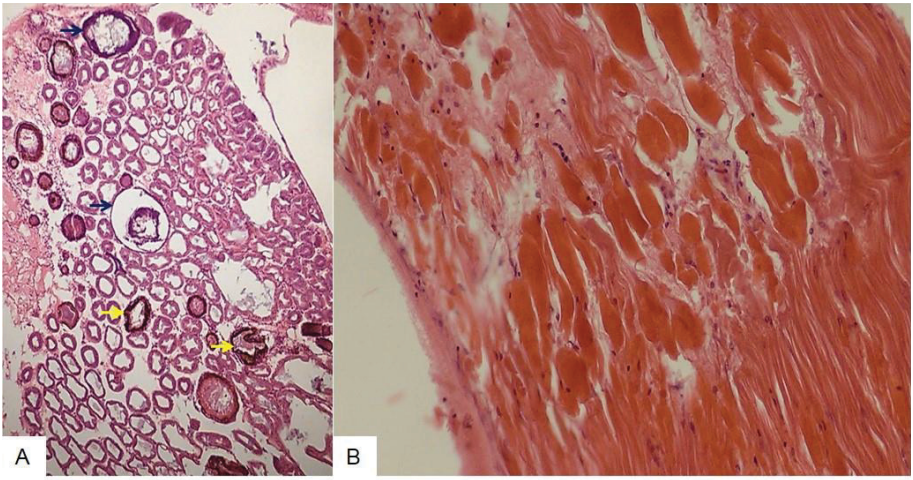
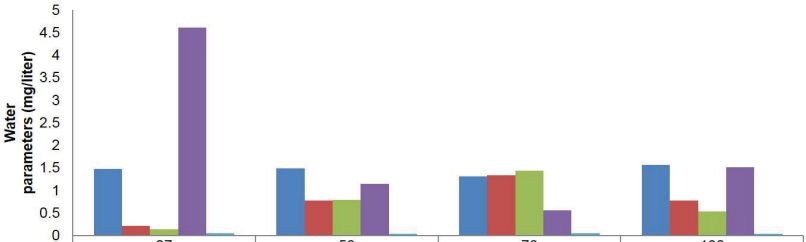


Fig. 3. (A). Hepatopancreatic cells with multifocal septic (black arrow) and melanized hepatopancreatic tubules with haemocyte encapsulation (yellow arrows) and (B). A focal acute necrosis with no obvious agent associated with the lesions in muscle.

Table 5. Water quality parameters in three farms in Perak compared with optimal water quality for shrimp culture.

Water Quality	Perak			Optimal water quality for shrimp culture
	Farm 1	Farm 2	Farm 3	
Temperature (℃)	31.0 – 31.1	31.7	–	25 – 30
Dissolved oxygen (mg.L^{-1})	8.7 – 9.6	8.5	3.5 – 5.5	> 4
pH	7.9 – 8.5	7.7	7.7 – 7.9	7.5 – 8.5
Salinity (ppt)	18.0 – 19.0	21.0	20.0 – 30.0	10.0 – 25.0
Nitrite (mg.L^{-1})	0.02 – 0.05	0.0	0.11	< 1.0
Un-ionized ammonia (mg.L^{-1})	3.9 – 5.0	4.8	3.65	< 0.1
Iron (mg.L^{-1})	0.3 – 1.0	0.7	0.5	–
Un-ionized hydrogen sulfide (mg.L^{-1})	29.0 – 52.0	65.0	33	< 0.005

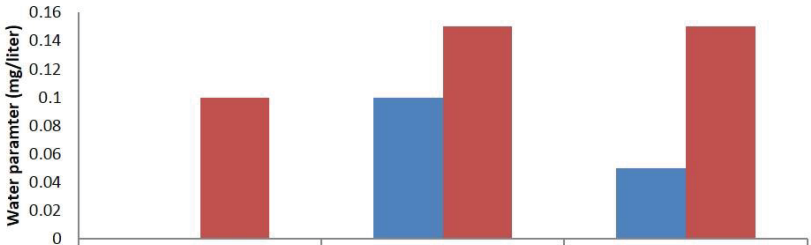


	27	58	76	138
NH3	1.47	1.49	1.31	1.57
NO2	0.21	0.78	1.33	0.77
NO3	0.134	0.786	1.438	0.53
PO4	4.61	1.15	0.56	1.51
S	0.047	0.031	0.052	0.039

Fig. 4. Cross-sectional study on chemical parameters of water quality in different day of culture (DOC) in one farm in Perak.

Table 6. Cross-sectional study on chemical parameters of water quality in different days of culture (DOC) in five farms in Perak.

Day of culture (DOC)	Farm	Pond	Water quality parameters (ppm)				
			Ammonia	Nitrate	Nitrite	Phosphate	Sulfide
32	1	B4	1.27	0.9	0.58	1.83	37
33	2	AA	2.06	0.41	2.96	1.88	40
42	3	2	1.14	0.27	0.19	1.46	39
58	4	A3	1.2	1.58	1.46	0.16	53
68	5	B3	1.31	0.08	0.04	1.32	70



	8 DOC	15 DOC	24 DOC
NH3	0	0.1	0.05
NO2	0.1	0.15	0.15

Fig. 5. Chemical parameters of water quality in a pond cultivating *Penaeus vannamei* in Perak.

Shrimp Sampling in Pahang and Penang States

Samples from Pahang and Penang were processed similar to samples from Perak. All shrimp samples (100 %) showed whitish patches in the abdominal segments, and 80 % had pale hepatopancreas, soft body and empty gut. All samples came from ponds with a history of mortality at 50 DOC except those from the hatchery at Balik Pulau, Penang. Haemolymph clotting time in 50 and 60 % of the shrimp from Pahang and Penang, respectively, exceeded 1.5 min, showing that they were under stress (Table 3). No farm tested positive for IHHNV infection. Pahang samples showed bacterial infection (*Vibrio* spp. and *Photobacterium damsela*). Samples from Pahang and Penang showed similar pathology in the hepatopancreas typical of developing EMS. However, in samples from Pahang, haemocytic infiltration and necrosis in the abdominal muscles were observed. This pathology is similar to lesions observed in IMNV or *Penaeus vannamei* nodavirus (PvNv). However, tests by IQ Plus for IMNV and PvNvin tissues obtained from live samples were negative (Table 4).

Discussion

The haemolymph clotting time test indicated 85, 50 and 64 % of the sampled shrimp were under stress in Perak, Pahang and Penang, respectively. Such stress could be triggered by disease occurrence, drastic changes in water quality parameters, poor diet or improper management. All of these factors, especially poor water quality, were present in all the investigated ponds. Stress due to consistent exposure to a high ammonium level during culture could be a contributing factor. Salinity, pH and dissolved oxygen (DO) levels were within acceptable ranges for shrimp culture, except for ammonium, nitrite and phosphate (Cheng et al. 2003). Throughout the cross-sectional study, the mean of ammonium concentrations ranged from 1.2–2.0 ppm, levels that were higher than the optimal range for cultured shrimp. Chien (1992) reported that ammonia is toxic to shrimp at high concentration, and Kasnir et al. (2014) highlighted that most shrimp can tolerate ammonia at a concentration of <0.1 $mg.L^{-1}$ NH_3–N. Data from one farm showed that at 27 DOC, rearing water for smaller shrimp has lower ammonium levels compared to that for larger shrimp at 58, 76 or 138 DOC, respectively. In another study, data from five farms registered higher ammonia levels ranging from 1.2–2.0 ppm, irrespective of the DOC at 32, 33, 42, 58 and 68. The increase in NH_4^+-N concentrations at certain times over the cultivation period could be due to the increased size of shrimp and the feeding rates (Guerrero-Galván et al. 1999).

At shorter DOC or smaller size, shrimp exposed to higher ammonium levels may not be able to tolerate such stress, which could reduce their immunity to infection. We believe that larger shrimp or those cultured for a longer period (i.e. > 40 DOC) are able to cope well compared with those at a shorter DOC (i.e. < 40). Prolonged exposure to stress due to a high concentration of ammonium could inhibit shrimp growth, as it could cause deterioration of the hepatopancreas and subsequently lead to increased susceptibility to EMS or AHPND.

Besides being associated with prolonged stress due to high concentrations of ammonium, EMS also could have another toxic etiology. Lightner et al. (2012) highlighted that degenerative pathology of the hepatopancreas is frequently a result of toxin. However, laboratory experiments conducted by Lightner et al. (2012) on crustacide and commercial feeds did not produce a consistent result similar to EMS pathology. The present study showed the presence of PSP toxin in infected shrimp; however, the concentration of toxin was lower than the human lethal dose of 2 mg (Hwang et al. 1992). The presence of PSP toxin in organs indicates that the affected shrimp ingested the toxin through food web transfer. The possibility of the toxin being present in commercial feeds, in common bacteria in the environment or in plankton could thus not be ruled out. In another study, Furio et al. (2012) showed that among several PSP-causative species of *Alexandrium*, *A. minutum* was found in low-salinity brackish environments in Viet Nam, Thailand, the Philippines and Malaysia. We believe that the presence of PSP toxin in EMS-affected shrimp could come the ingestion of diatoms, dinoflagellates or other micro-organisms in the water.

As the affected shrimp were under stress, we believe that they were more susceptible to all kinds of common pathogens, and particularly susceptible to multiple bacterial infections by members of the *Vibrio* group. Under these conditions, the shrimp were also exposed to other living organisms, including the dinoflagellate, which showed some PSP toxin. There is also the possibility that the toxin seen in affected shrimp showing acute pathology of the hepatopancreas could be protein based, as that toxin can be detected by the ELISA method used in the present study.

During the investigations conducted during Phases I and II (see Table 1), we observed that all samples tested for IMNV were negative, indicating that IMNV was not the cause of mortality despite the appearance of whitish abdominal muscles. According to the farm operators, most of the PL used originated from SPF broodstocks, and the affected shrimp tested negative for IMNV and TSV before being stocked into the ponds. Most of the samples also tested negative for IHHNV, with only seven of 64 samples from Perak being positive. IHHNV infection is known to cause "runt deformity syndrome", irregular and reduced growth, and cuticular deformities in *P. vannamei* (Kalagayan et al. 1991; Brock and Main 1994; Lightner 1996). During the investigation period, a single positive case of IHHNV in the affected shrimp would not have caused high mortality in cultivated *P. vannamei*. Vibriosis was recorded in samples from Perak and Pahang, but not in Penang. *Vibrio* species are part of the natural microflora of wild and cultured shrimp (Sinderman 1990) and become opportunistic pathogens when the natural defense mechanisms of shrimp are suppressed (Brock and Lightner 1990).

Mortalities due to vibriosis also occur when shrimp are stressed by factors such as poor water quality, crowding, high water temperature, low DO and low water exchange (Lewis 1973; Lightner and Lewis 1975; Brock and Lightner 1990). Vibrios are among the most important bacterial pathogens found in cultured shrimp, and they are responsible for a number of diseases

where mortalities may be up to 100 % (Jayasinghe et al. 2008). Shrimp-pathogenic vibrios are mainly *V. harveyi*, *V. fluvialis, V. parahaemolyticus*, *V. damsela* and *V. vulnificus* (Chythanya and Karunasagar 2002). The present study revealed that multiple bacterial infection consistently showed the presence the of *V. parahaemolyticus* in affected the shrimp.

Histopathological analysis showed a typical histopathology of AHPND in the hepatopancreatic tubules. Karyomegaly, sloughing of epithelial cells from hepatopancreatic tubules, multifocal septic tubules and melanized hepatopancreatic tubules with haemocyte encapsulation were seen in some specimens, suggesting the acute and terminal stages of AHPND. This provides evidence that the disease outbreaks in shrimp ponds in Perak, Pahang and Penang were due to AHPND.

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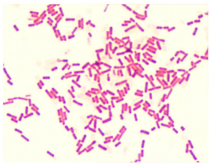
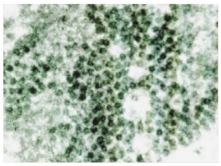
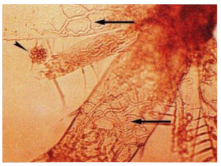
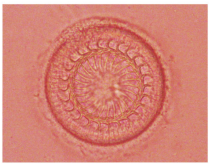

Aquatic Emergency Preparedness and Response Systems for Effective Management of Transboundary Disease Outbreaks in Southeast Asia (AEPRS)

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Eleonor A. Tendencia
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Emergency Preparedness and Response Systems for Aquatic Animal Diseases in Malaysia

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Abstract

The Department of Fisheries (DoF) Malaysia is the custodian of the Fisheries Act 1985, which serves as the main legislative source for subsidiary regulations, including aquaculture and fish health management. It has established Emergency Disease Task Force Committee for any emergency related to disease outbreak as well as standard operating procedures for massive fish kill. This committee consists of taskforce teams from federal and/or state fisheries and oversee the operations of the task force. Fisheries Biosecurity Division under DoF Malaysia holds the primary responsibility for managing the country's emergency preparedness and response system for aquatic animal diseases. As for early detection system, Fisheries Biosecurity Division has established official control and official analysis for targeted diseases listed under OIE and National Listed Diseases. Fish health monitoring programmes are conducted every six months and samples are analyzed by accredited laboratories. Quarterly and half year reports are submitted to representative offices for the health status of targeted disease. Apart from the targeted fish health monitoring program, epidemiology on common and emerging diseases are conducted by National Fish Health Research Division (NaFisH) which is the only research and development arm under DoF. Laboratories under Fisheries Biosecurity Division are responsible for organizing and coordinating surveillance programs for diseases in the OIE list while NaFisH is responsible for conducting research and development on aquatic diseases that cause high losses in industry since 2002. Currently, the DoF has four servicing laboratories under Fisheries Biosecurity Division and one NaFisH laboratory under Fisheries Research Institute for fish health diagnosis in Malaysia.

Keywords: Aquatic Health, Emergency Preparedness, Response Systems, Malaysia

Introduction

Aquaculture in the Malaysia has grown dramatically and continued to show a rapid growth. The amount of fish demand is expected to increase from 1.3 million tons in 2010 to 1.9 million tons in 2020 with growth of 3.8% per year. Per capita consumption of fish is expected to increase from 20 to 55 kilogram with growth of 1.9% annually. Aquaculture production is projected to increase to 790,000 metric tons, equivalent to 41% of total demand state fish in 2020. Export value of aquaculture, including fish products especially fillet, is expected to increase from RM1.4 billion in 2010 to RM3.2 billion in the year 2020. From 2016 to 2017, fish production from aquaculture grew 5% per year (DoF Malaysia 2016 and 2017). In terms of commodities, seaweeds contributed 47.5% from the total aquaculture production in 2017, followed by Hawaiian white shrimp (8.3%), freshwater catfish (8.2%), sea bass (7.1%), red tilapia (6.0%) and pangasius (4.7%) (Figure 1).

As one of the fast growing industry in Malaysia, aquaculture sector also faced challenges related to various aquatic animal issues, managing or untimely response to disease emergencies such as disease outbreak, mass mortalities, emerging or re-emerging diseases. In order to fulfil the requirements of increased production and to secure food security for long-term sustainability, Department of Fisheries (DoF) Malaysia has been focusing on efforts to improve the quality, efficiency and effectiveness of service delivery and partnerships between the DoF and stakeholders. The DoF is the Competent Authority (CA) for fish

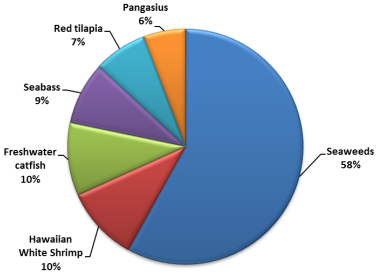


FIGURE 1. Growth of Aquaculture in 2017, Malaysia

health and biosecurity management in Malaysia (Figure 2). The CA manages fish health based on main legislative acts for subsidiary regulations, including aquaculture and fish health management. The relevant legislation implemented in Malaysia are the Fisheries Act 1985, Malaysian Quarantine And Inspection Services Act 2011, Feed Act 2009 and Animal Welfare Act 2015. As for East Malaysia, addition regulation such Inland Fisheries and Aquaculture Enactment 2003 has been implemented by DoF Sabah as well as State Fisheries Ordinance 2003 by Department of Agriculture (DoA) Sarawak (Table 1). The relevant government departments use the legislation as guidelines, and through detailed discussion with stakeholders, to formulate mechanisms that are standardised and suit the needs of industry and international trade. The implementation required rapidity and effectiveness on government to recognise and react to the first report of serious disease through early warning, first detection and responding system.

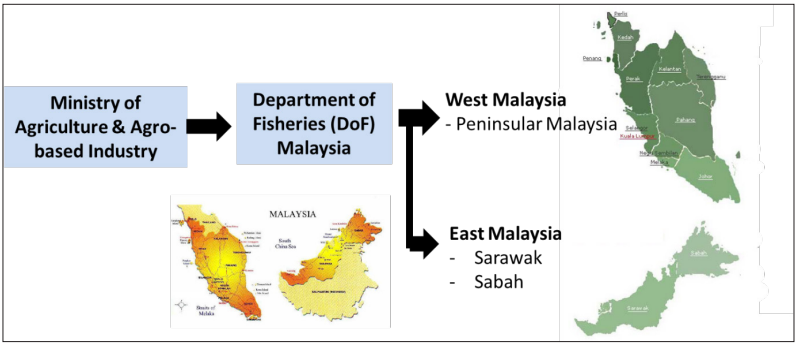


FIGURE 2. National Government Agency (CA) of fish health in Malaysia

TABLE 1. Legislative acts on fish health implemented in Malaysia

Act	Regulation
Fisheries Act 1985 (Act 317)	• Fisheries Regulation 1990 (Marine Culture System) • Fisheries Regulation 2002 (Cockle Culture and Conservation) • Fisheries Regulation 2009 (Quality of Fish for Export to the European Union) • Fisheries Regulation 2012(Fish Disease Control Compliance for Exports & Imports) • Fisheries Regulations 2010 (Prohibition of Import, etc, of Fish) (Amendment 2011) • Fisheries(Inland Fisheries Aquaculture) (Federal Territory of Kuala Lumpur and Federal Territory of Labuan) Rules 2017
Malaysia Quarantine And Inspection Services Act 2011(Act 728)	• Malaysian Quarantine and Inspection Services Regulations 2013 (Quarantine and Inspection) • Malaysian Quarantine and Inspection Services Regulations 2013 (Quarantine Procedures) • Malaysian Quarantine and Inspection Services Regulations 2013 (Issuance of Permit, License and Certificate) • Malaysian Quarantine and Inspection Services Regulations 2013 (Registration of Importers, Exporter and Agents) • Malaysian Quarantine and Inspection Services Regulations 2013 (Fees and Charges)
Feed Act 2009 (Act 968) Section 53(2) (b), (c), (e), (f), (g) and (h)	• Feed (License to Import Feed and / or Feed Additive) Regulations 2011 • Feed (Labelling of Feed and Feed Additive) Regulation 2011 • Feed (Prohibited Use of Antibiotics, Hormones or Others Chemicals) Regulation 2011 • Feed (Manufacture and Sale of Feed and Feed Additive) Regulation 2011 • Feed (Methods of Analysis of Feed and the Form of Certificate of Analysis) Regulation 2011
Inland Fisheries and Aquaculture Enactment 2003 of Sabah State	• Part IV - Aquaculture • Part VI - Control of Fish • Part VI - Control of Fish Diseases • Part X - Enforcement
Law of Sarawak, Chapter 54, State Fisheries Ordinance 2003	• Part VI - Control of Fish Diseases • Part VII - Fish Products and Fish Processing • Part VIII - Enforcement

Early Warning System

DoF Malaysia has established Emergency Disease Task Force Committee (EDTFC) which acts as national aquatic emergency preparedness and response committee toward any emergency related to disease outbreak as well as standard operating procedures for massive fish kill. This committee led by Fisheries Director General and cover Fishkill Task Force Committee and State Task Force Committee. The main tasks are to monitor, provide guidance, evaluate, oversight of progress and assist in key decisions regarding the implementation of task force.

Information from national aquatic epidemiology, alerts news from DoF Malaysia Corporate Communications Unit (CCU), National Aquatic Animal Health Focal Point (NAAHFP) for OIE and reports from DoF staff serve as early warning system for DoF Malaysia particularly to EDTFC (Table 2). The national authority monitors aquatic animal disease events in other countries through internet, literature search and attending regional consultation meetings, seminar, symposium, conference or workshop. CCU will gather news related to fisheries through social media while NAAHFP will receive latest notification of any new diseases from OIE and NACA website and subsequently will alert DoF. DoF staff participating in the regional consultation meetings, training, seminar, symposium and conference will prepare a

TABLE 2. National information sharing networks

Network	Information sharing
Corporate Communications Unit (CCU) under DoF	**Social media (Facebook, Twitter, Instagram, Whatsapp)** • Fisheries related news/issues
National aquatic animal health focal point (NAAHFP) for OIE - *E-network Malaysian Aquatic Animal Health Expert (MAAHE)*	**E-mail** • Quarterly Aquatic Animal Disease Report (QAAD) • Aquatic Animal Disease Report (OIE) • The Aquatic Animal Scientific Commission Report (OIE)
Industry Consultation	**Dialogue and meetings** • Specific issues • New regulation/requirement
Farmers Day	**Seminar and dialogue** • Annual event organized by state

detailed report and alert DoF on immediate action if required. If the alert news can cause impact to industry, Fisheries Biosecurity Division will notify the EDTFC to take appropriate action (Figure 3). Currently, DoF Malaysia is developing a specific system regarding fish health information. Under this system, information on Official Control which covers detailed profiles and activities of stakeholders, fish disease notifications, reporting and mapping will be made available. As for Official Analysis, it will include information on the disease surveillance programme and laboratory analysis. This system will be ready for use at DoF headquarter and at the state level in coming year.

DoF Malaysia also conduct risk analysis to identify high priority aquatic disease threats for introduction of alien aquatic species. Import Risk Analysis (IRA) covers list of diseases, biodiversity or genetic threat to national aquatic resources which will be carried out during the application process.

Early Detection and Response System

DoF Malaysia has developed a Fish Disease Notification Form that has been distributed to registered farms/premises (Figure 4). All registered farms/premises are obliged to notify DoF in case of the occurrence or suspicion of a listed fish disease or the occurrence of mass mortality. Farmers, state aquaculture or biosecurity fishery officers act as front line and continue to receive training and awareness on fish health management from time to time.

Apart from EDTFC, DoF Malaysia also established national aquatic epidemiology or on-ground aquatic animal disease management through Fisheries Biosecurity Division and National Fish Health Research Division (NaFisH). Fisheries Biosecurity Division is responsible for (1) preparing and drafting policies on fish and public health management, (2) providing laboratory services on fish disease diagnostics and food safety, (3) implementing the development of fish and fisheries product standards at national and international level, (4) coordinating on capacity building of staff and their training on relevant fields, and (5) managing the administration and financial aspect of the Fisheries Biosecurity Division. On their hand, NaFisH responsibilities included (1) conducting and implementing research and development of aquatic animal health specifically on fish, shrimp and mollusc health management, (2) providing laboratory services on

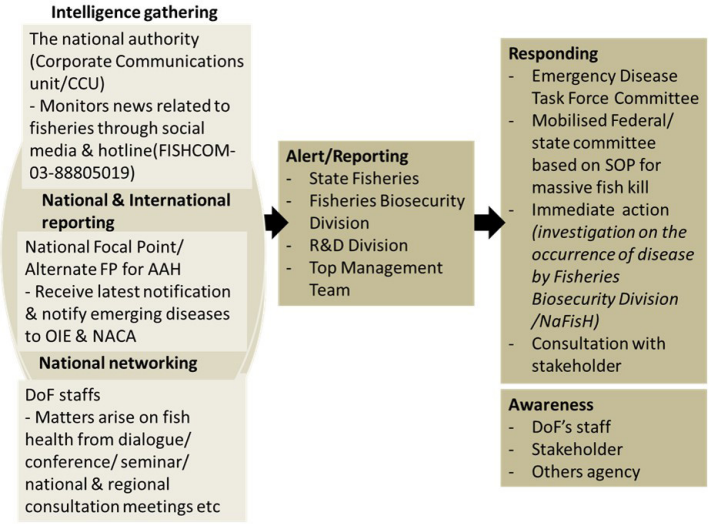
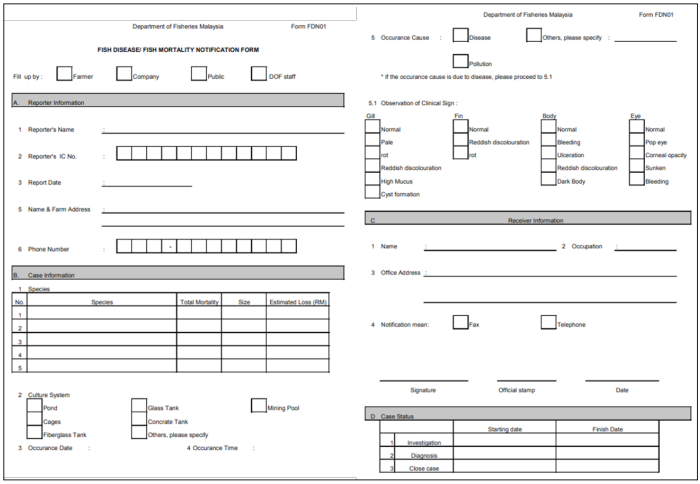


FIGURE 3. Mechanisms on early warning system by national authority on fish health management



Department of Fisheries Malaysia Form FDN01

FISH DISEASE/ FISH MORTALITY NOTIFICATION FORM

Fill up by : Farmer Company Public DOF staff

A. Reporter Information

1 Reporter's Name :

2 Reporter's IC No. :

3 Report Date :

5 Name & Farm Address :

6 Phone Number :

B. Case Information

1 Species

No.	Species	Total Mortality	Size	Estimated Loss (RM)
1				
2				
3				
4				
5				

2 Culture System

Pond Glass Tank Mining Pool

Cages Concrate Tank

Fiberglass Tank Others, please specify

3 Occurance Date : 4 Occurance Time :

Department of Fisheries Malaysia Form FDN01

5 Occurance Cause : Disease Others, please specify :

Pollution

* If the occurance cause is due to disease, please proceed to 5.1

5.1 Observation of Clinical Sign :

Gill	Fin	Body	Eye
Normal	Normal	Normal	Normal
Pale	Reddish discolouration	Bleeding	Pop eye
rot	rot	Ulceration	Corneal opacity
Reddish discolouration		Reddish discolouration	Sunken
High Mucus		Dark Body	Bleeding
Cyst formation			

C Receiver Information

1 Name : 2 Occupation :

3 Office Address :

4 Notification mean: Fax Telephone

Signature Official stamp Date

D Case Status

		Starting date	Finish Date
1	Investigation		
2	Diagnosis		
3	Close case		

FIGURE 4. Fish Disease Notification Form

fish disease diagnostics and technical assistance in fish health management to farmers, (3) providing training on fish health managements to DoF staffs and those concerned and (4) acting as adviser in main committee of National Fish Health Strategy and EDTFC. Both divisions will provide awareness and announcement through dialogs/forum to the target groups on any emerging new cases especially on the disease impact, occurrence of disease in the neighbouring countries as well as the control measures and actions to be taken by target groups (associations, breeders and other traders).

The difficulties in handling and managing the disease problems in aquaculture system are well known and worsen by the uncontrolled movement of aquatic animal species through global trading. Thus, there is a need of the industry to be aware of the current status and issues in aquatic animal health and diseases with regards to local and international requirements. In view of the increasing need of the Aquaculture industry, DoF established Fish Health Research unit under Fisheries Research Institute in 1996 with a focus on development of national personnel level with expertise on aquatic animal health. Since then, the unit grown rapidly and in 2002, the unit was upgraded into a center, carrying out R&D on fish diseases programmes, developing database of epizootics for early warning of diseases while providing and enhancing the capacity for diagnostics and disease prevention. Through five epidemiology projects focusing on diseases at national level, a database on National Pathogen Lists was established in 2010 and since then, disease surveillance on common and emerging diseases studies are based on those that cause high economic losses in the country.

Since 2010 onward, Fisheries Biosecurity Division has established official control and official analysis for targeted diseases listed under OIE-listed diseases and National Pathogen Lists (Table 3). Surveillance programme for fish, shrimp and mollusc diseases were established (Table 4). Fish health monitoring programme were conducted every six months under accredited laboratories. Currently, DoF has four servicing laboratories under Fisheries Biosecurity Division and one National Fish Health Research Division laboratory under Fisheries Research Institute for fish health control in the whole of Malaysia. These laboratories are responsible for testing of samples from the disease surveillance and investigation of fish mass mortality cases (Figure 5). From time to time, capabilities of DoF are enhanced through training conducted by national and international bodies. In the case of TiLV, two staffs of DoF were sent for TiLV course in Thailand in 2017. At the same time, development of RT

PCR detection method for TiLV was established at NaFisH. The national laboratories of Fisheries Biosecurity Division have knowledge in organising and coordinating surveillance for diseases in the OIE list while laboratories under NaFisH have been organising and coordinating surveillance for diseases that cause high losses in the country. Currently, all DoF personnel had gone through basic training course, Diagnostics Level I, II and III on aquatic animal health according to The Asia Diagnostic Guide (Melba et al., 2001).

Quarterly and half year reports were prepared by Fisheries Biosecurity Division and validation was carried by NAAHFP before submitting to representative offices for the health status of targeted disease (Figure 6). For emerging diseases, confirmation diagnosis test under national competent authority will be carried and followed by notification to OiE by NAAHFP. DoF Malaysia works hand in hand with others agencies such as (a) Department of Environment Malaysia for reporting, sampling and investigation in mass mortality of fish in open water, (b) Department of Veterinary Services (DVS) for notification/ reporting to OIE/NACA, and (c) Department of Chemistry for further laboratory analysis of unexplained mortality in open water. The positive cases were disposed under the supervision of DoF.

TABLE 3. Targeted diseases that listed under OIE-listed diseases and National Pathogen Lists

OIE-listed Diseases	National-listed Disease	Importing Country Requirements
Finfish • Koi Herpes Virus (KHV) • Spring Viraemia of Carp (SVC) • Red Sea bream Iridovirus (RSIV) • Epizootic Ulcerative Syndrome (EUS) • *Gyrodactylus salaris*	• Viral Nervous Necrosis (VNN) • Iridovirus • *Streptococcus* sp. • Enteric Septicemia of catfish • Nocardiosis • Flexibacter • Vibriosis • *Gyrodactylus* sp. • Skin monogenean • Isopod infestation	• Megalocytivirus • *Aeromonas salmonicida* (AS) • Enteric Redmouth Disease (ERD)
Shrimp • White Spot Syndrome Virus (WSSV) • Infectious Myonecrosis Virus (IMNV) • Infectious Hypodermal and Haemapoietic Necrosis Virus (IHHNV) • Taura Syndrome Virus (TSV) • Yellowhead Virus (YHV) • Macrobrachium Nodavirus (MRNV) • Acute Hepatopancreatic Necrosis Disease (AHPND)	• *Enterocytozoon hepatopenaei* (EHP) • Hepatopancreatic Parvovirus (HPV) • Spherical Baculovirus	
Mollusc • *Perkinsus olseni* • *Perkinsus marinus*	• *Perkinsus* spp.	

TABLE 4. Type of surveillance conducted in Malaysia

Active Surveillance	Passive Surveillance
Shrimp • Yellow Head Virus (YHV) • Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV) • Infectious Myonecrosis Virus (IMNV)	**Shrimp** • *Enterocytozoon hepatopenaei* (EHP) • Hepatopancreatic Parvovirus (HPV) • Acute Hepatopancreatic Necrosis Disease (AHPND) • Spherical baculovirosis (*P. monodon*-type baculovirus)
Fish • Koi Herpesvirus (KHV) • Spring Viraemia of Carp (SVC) • Red Seabream Iridovirus • Epizootic Ulcerative Syndrome • Megalocytivirus • *Aeromonas salmonicida* • Viral Nervous Necrosis	**Fish** • Streptococcosis • Enteric Septicimia of Catfish • Vibriosis • Capsalid (Skin monogenean) infestation • *Gyrodactylus* infestation • Mycobacteriosis • Isopod infestation • Tilapia Lake Virus

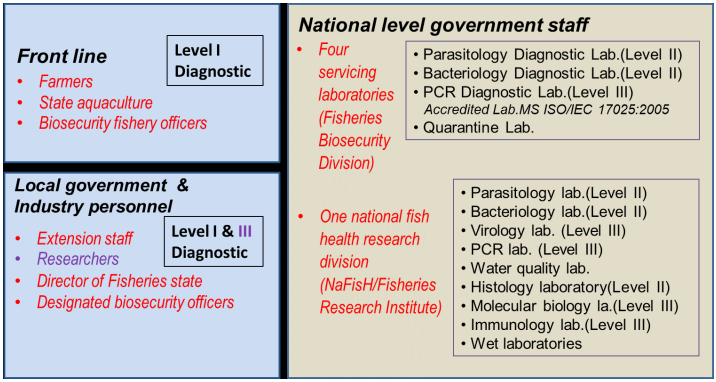


FIGURE 5. Personnel competencies of national competent authority in Malaysia

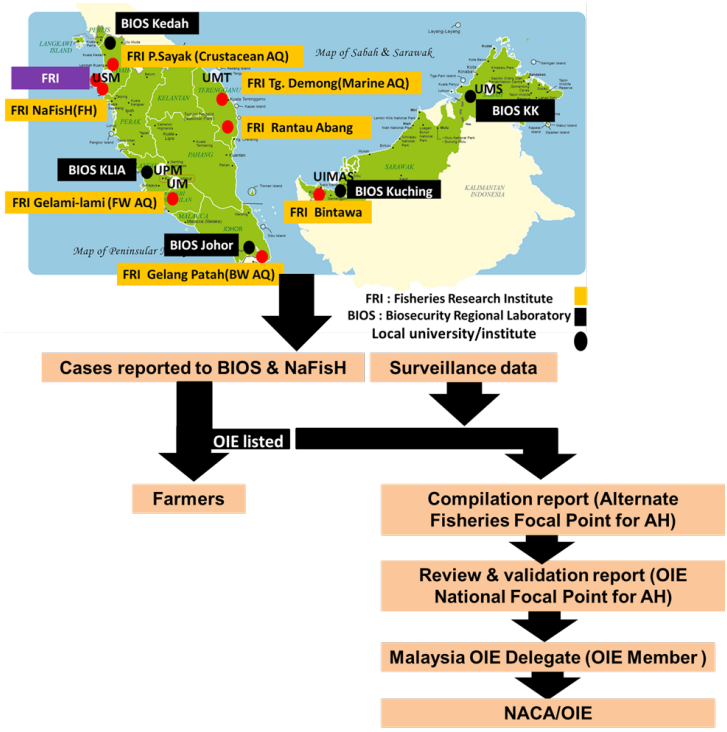


FIGURE 6. Flow chart of aquatic animal health reporting system in Malaysia

Summary

Fisheries Biosecurity Division is responsible for the implementation of official control, official guarantee and official analysis for fish and fishery products along the supply chain from farm to the exporter premises. These responsibilities cover Peninsular Malaysia, and the states of Sabah and Sarawak on the island of Borneo.

Using current procedure for early warning system, the national competent authority on fish health management was implemented on emerging disease of Acute Hepatopancreatic Necrosis Disease (AHPND) in 2011 (Figure 7) and Tilapia Lake Virus (TiLV) in 2017 (Figure 8). Imposing IRA as compulsory for country also prevent the introduction of alien aquatic species which may pose threat to national aquatic resources. Since 2010, DoF had processed and conducted 51 IRA applications for bringing in non-indigenous species to Malaysia and only 21 applications were approved (Figure 9). Through early detection and response system, an average of 15 active surveillances were performed on average of 465.4 registered farms (Figure 10). Between 2012 and 2016, the status of targeted diseases were identified (Figure 11) and the information obtained helps the competent authority to establish new guidelines for improving better fish health management.

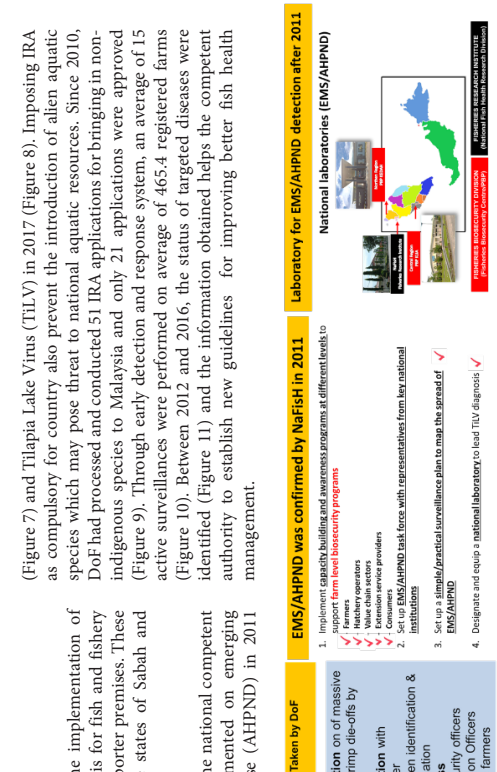


FIGURE 7. National news mechanisms for emergency preparedness and response system for emerging disease of AHPND in 2011

FIGURE 8. National/International reporting for emergency preparedness and response system for emerging disease of Tilapia Lake Virus (TiLV) in 2017

FIGURE 9. Status of application for bringing in non-indigenous species to Malaysia after IRA

Legend:
KHV - Koi Herpesvirus, SVC - Spring Viraemia of Carp, AS - *Aeromonas salmonicida*, EUS - Epizootic Ulcerative Syndrome, TiLV - Tilapia Lake Virus,
GY - *Gyrodactylus salaris*, WSSV - White Spot Syndrome Virus, IHHNV - Infectious Hypodermal & Hematopoietic Necrosis Virus, IMMV - Infectious Myonecrosis Virus,
YHV - Yellow Head Virus, TSV - Taura Syndrome Virus, AHPND - Acute Hepatopancreatic Necrosis Disease, RSIV - Red Sea Bream Iridovirus, PER - *Perkinsus olseni*, and VNN - Viral Nervous Necrosis

FIGURE 10. Fish Disease Surveillance Programmes from 2012 until 2018

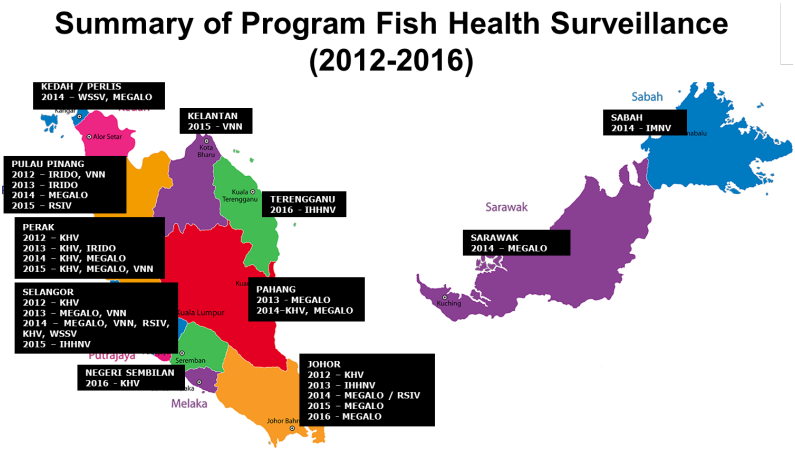


FIGURE 11. The summary of fish health programme between 2012 and 2016

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ADDRESSING ACUTE HEPATOPANCREATIC NECROSIS DISEASE (AHPND) AND OTHER TRANSBOUNDARY DISEASES FOR IMPROVED AQUATIC ANIMAL HEALTH IN SOUTHEAST ASIA

Rolando V. Pakingking Jr.
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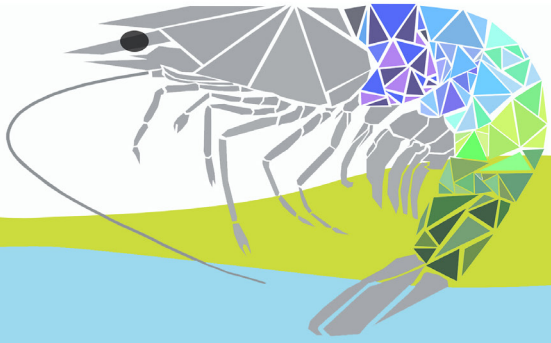
Editors



Japan-ASEAN Cooperation

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Current Status of Acute Hepatopancreatic Necrosis Disease (AHPND) of Farmed Shrimp in Malaysia

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Abstract

A report about a disease problem in cultured whiteleg shrimp (*Penaeus vannamei*) was first received by the National Fish Health Research Center (NaFisH) in 2011 from Perak State showing signs of white feces and slow death leading to serious mortality rate. Later, in September of the same year, the Malaysian Shrimp Farmers Association (MSFA) reported to Department of Fisheries (DOF) severe mortalities in almost all of the whiteleg shrimp farms throughout Peninsular Malaysia. Sampling of shrimps for disease diagnosis was then conducted by NaFisH. The bacteriological and histopathological examinations revealed respectively the isolation of *V. parahemolyticus* and massive sloughing of hepatopancreatic epithelial cells. The disease was subsequently identified as acute hepatopancreatic necrosis disease (AHPND). From our 3-year study, the annual prevalence rates of AHPND were 50%, 26% and 73% in 2011, 2012 and 2013, respectively. At present, AHPND still persists in Malaysia but at a lower prevalence. The risk factors associated with the disease were studied, however, varied environmental and management data analyzed were inconclusive to relate any one parameter directly to the disease. To help ensure the early detection of AHPND, an experimental observation study on `gut scorecard' was carried out and this was confirmed by PCR and histopathology. Validation of this technique has yet to be carried out to ensure its reliability. We also examined the potential use of some commercial products such as probiotics and disinfectants available in the market but unfortunately results showed that they were not effective in controlling AHPND. Control measures applied by the farmers such as the use of probiotics were also verified but data generated likewise appeared to be inconclusive. On the contrary, our preliminary study on the antibacterial property of the plant extracts, i.e. betel and lemongrass, incorporated in the feed showed some prophylactic and chemotherapeutic potential against AHPND. However, comprehensive *in vitro* and *in vivo* trials are still currently being undertaken to elucidate its efficacy and practical applications. To ensure the shrimp industry's sustainability in Malaysia, results of our ongoing and future studies aimed at preventing and controlling unwarranted outbreaks of AHPND and other emerging transboundary diseases of penaeid shrimps will be continually disseminated to shrimp farmers and pertinent stakeholders.

Introduction

The whiteleg shrimp (*Penaeus vannamei*) is a popular choice species for shrimp farmers in Malaysia. Whiteleg shrimp culture started in Peninsular Malaysia in 2002. The contributions of whiteleg shrimp and tiger prawn (*P. monodon*) in annual production of farmed shrimps were 70% and 30%, respectively in 2007 (Annual Fisheries Statistics, 2005-2014). They have been successfully cultured in earthen ponds. However, in the latter part of 2010 and early part of 2011, farmers in the southern and middle regions of Peninsular Malaysia encountered several disease outbreaks during the early stage of shrimp culture. At that time, the outbreak was known as early mortality syndrome (EMS) or acute hepatopancreatic necrosis syndrome (AHPNS). Early mortality syndrome was later named as acute hepatopancreatic necrosis disease (AHPND) in 2013 (Tran *et al.*, 2013). AHPND is caused by a unique strain of *Vibrio parahaemolyticus* capable of releasing potent toxins that could consequently lead to tissue destruction and dysfunction of the hepatopancreas (GAA, 2013).

Outbreaks of AHPND among the major whiteleg shrimp-producing farms located in five major states in Peninsular Malaysia had consequently resulted in serious economic losses estimated at USD 0.1 billion in 2011 (NACA 2012). In the course of our investigation, i.e. from 2011 to 2013, we found out that AHPND had caused 40 to 100% mortality of cultured whiteleg shrimps. We also discovered that some surviving whiteleg shrimps had slow growth that subsequently succumbed to morbidity and mortality at the latter stage of culture. Occurrence of AHPND has been reported in China (2009), Viet Nam (2011), Thailand (2012) and the Philippines (2015) (Tran *et al.*, 2013; Joshi *et al.*, 2014; dela Peña *et al.*, 2015). Apart from these Asian countries, AHPND has also been reported in Mexico in 2013 (Lightner *et al.*, 2013; Soto-Rodriguez *et al.*, 2015). AHPND has caused significant reduction in the world shrimp production (NACA, 2013; FAO, 2013; Lightner *et al.*, 2012; Leaño and Mohan, 2012).

In Malaysia, investigations on AHPND episodes since 2011 have been accordingly categorized into 5 phases. Phase I was conducted in 2011 with the main objective of identifying the etiology of the disease. Phase II on the other hand, delved on factors associated with AHPND outbreaks. Moreover, Phase III focused on control and preventive measures. Finally, Phases IV and V have been concurrently conducted to generate substantial data on the epidemiology of AHPND, establish practical and accurate methods for the early detection of AHPND at the farm level, and develop practical and effective preventive and therapeutic methods through the use of herbs or environment friendly products with potent antibacterial properties.

History of occurrence

In the mid 2011, the Fisheries Research Institute through the National Fish Health Research Division (NaFisH) received reports of two cases of cultured whiteleg shrimps exhibiting white feces and slow death from shrimp operators in Perak State. Subsequently, the Department of Fisheries (DOF) of Malaysia was informed by the Malaysian Shrimp Farmers Association (MSFA) of high mortalities at alarming rates in most of the shrimp farms located throughout Peninsular Malaysia in September 2011. An immediate action was undertaken to determine the possible cause or etiology of the outbreak. Water and shrimps from affected areas were sampled and diagnosed for diseases. Relevant water quality parameters were also determined. During the course of our investigation, we discovered that the outbreak actually occurred in the east coast of Johor in the latter part of 2010. However, no samples were obtained from Johor during that period because before the sampling was about to be carried out, farmers had already done an emergency harvest. In 2011, the affected States included Perak, Penang, Kedah and Pahang. The etiology of the disease was later confirmed as AHPND based on histopathological changes in the hepatopancreas of affected shrimps and affirmation of the diagnostic finding by the

laboratory of Dr. D. Lightner in Arizona State University. Subsequently, samples from Sabah and Sarawak (2012), Terengganu (2013), Melaka and Johor (2014) States were also found positive for AHPND by histopathology (Figure 1).

Figure 1. Chronological order (year) of AHPND occurrences in Malaysia confirmed by histopathology

Severity and economic impact

Total production of cultured shrimp was 87,000 metric tons (MT) in 2010 and 90% of the production was contributed by whiteleg shrimps (Figure 2). Several outbreaks of AHPND in whiteleg shrimp farms in 2011, 2012 and 2013 resulted in the reduction of total shrimp production to 67,000 MT, 55,000 MT, and 50,000 MT in 2011, 2012, and 2013, respectively (Annual Fisheries Statistics, 2005-2014). Based on the estimated total shrimp production losses from 2011 to 2014, the total economic losses from AHPND episodes were estimated to reach USD 0.49 billion.

Species affected

Currently, two major species of farmed shrimps, i.e. whiteleg shrimp (*P. vannamei*) and tiger prawn (*P. monodon*), have been so far affected by AHPND resulting in serious mortalities. It was first detected in whiteleg shrimp and tiger prawn in 2011 and 2014, respectively. Infection rate was higher in whiteleg shrimp than tiger prawn. AHPND was documented in shrimp post larvae (PL) 7 to 12, juveniles, and broodstocks.

Disease signs and diagnostic methods

On-farm investigations of shrimps for AHPND diagnosis are usually conducted in farms experiencing mortality within 30 days of stocking shrimps in cultivation ponds or when large-scale die-offs commence. Shrimps infected with AHPND exhibit an array of clinical signs including lethargy, soft shells, whitish muscle, empty stomach and midgut, slow growth, and pale atrophied hepatopancreas that often have black streaks. However, these pond-level observations have to be further diagnostically confirmed as AHPND by taking into account

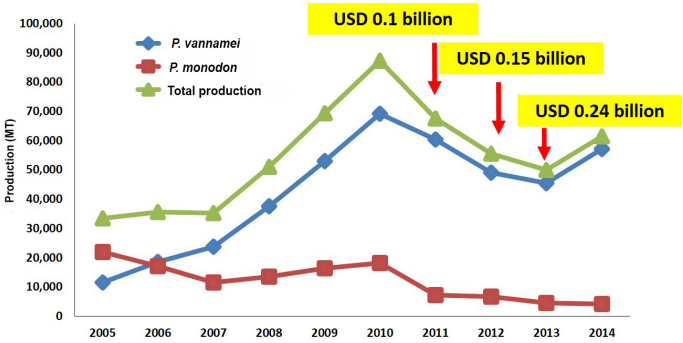

Figure 2. Production of farmed shrimps in Malaysia from 2005 to 2014 and corresponding losses estimated from the reduction of shrimp production.

the pathognomonic histopathological signs of AHPND including the isolation of *V. parahaemolyticus* (biochemically identified using API20E) and massive sloughing of hepatopancreatic tubule epithelial cells of the hepatopancreas of affected shrimps. From 2013 onwards, PCR assay using primers AP2, AP3 and AP4, Real-time PCR, and detection kit (IQ2000) have been employed as confirmatory tests for AHPND.

Status of AHPND

The current status of AHPND in farmed shrimps in Malaysia is chiefly based on the histopathological findings generated from the number of mortality cases reported to NaFisH, shrimp diagnostic laboratories, and epidemiological work carried out from 2011 to 2015. A total of 4,571 samples of farmed whiteleg shrimps and tiger prawns were obtained from 3 main diagnostic laboratories. The 3 main diagnostic laboratories receiving samples monthly include NaFisH, Fisheries Research Institute, and a private laboratory. The positive cases of AHPND in whiteleg shrimp were 50%, 26%, 34%, 13% and 4% in 2011, 2012, 2013, 2014 and 2015, respectively (Table 1). AHPND was also detected in tiger prawn in 2014 (10%) and 2015 (5%), respectively. At present, AHPND still persists in Malaysia but at a lower prevalence.

Future directions

Research and development focusing on the early detection of AHPND at the farm level and usage of alternative treatment such as the use herb extracts or environment-friendly products have been carried out since 2013. The risk factors associated with the disease have been also studied, however variations in environmental and management data revealed to be inconclusive to relate any one parameter directly to the occurrence of AHPND. To assist early detection of AHPND in cultivated shrimps, experimental observation study on 'gut scorecard' has been carried out together with PCR and histopathology as confirmatory tests. However, validation of this technique to ensure its validity and reliability is ongoing. Studies on control measures have been also conducted at the farm level through a collaborative effort with the farmers using existing commercial products but the results showed that these commercial products were ineffective in controlling AHPND. Also, control measures formulated by the farmers themselves such as the use of probiotics were analyzed but most of these methods were likewise found to be ineffective. However, our laboratory trial using the betel and lemongrass extracts incorporated in the shrimp's diet conferred protection against an experimental challenge with *V. parahaemolyticus* strains implicated in outbreaks of AHPND in Malaysia. However,

Table 1. Samples of whiteleg shrimps and tiger prawns examined for AHPND from 2011 to 2015.

Year	Whiteleg shrimp (*P. vannamei*)			Tiger prawn (*P. monodon*)		
	Number of samples examined	Number of AHPND positive	Prevalence (%)	Number of samples examined	Number of AHPND positive	Prevalence (%)
2011	394	197	50.0			
2012	584	151	25.9			
2013	661	212	34.4			
2014	1,586	199	12.5	50	5	10.0
2015	1,346	50	3.7	74	4	5.4

more comprehensive studies still need to be conducted to elucidate its efficacy and practical applications. Aside from the imposition of stringent biosecurity measures in hatcheries and grow-out facilities, to realistically ensure the sustainability of the shrimp industry in Malaysia, results of our ongoing and future studies aimed at preventing and controlling unwarranted outbreaks of AHPND and other emerging transboundary diseases of penaeid shrimps will be continually disseminated to shrimp farmers and pertinent stakeholders.

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Antimicrobial Activities of Commercial Essential Oils Against V*ibrio parahaemolyticus* from Acute Hepatopancreatic Necrosis Disease of White Shrimp (*P. vannamei*)

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Abstract: Antimicrobial activities of five commercial essential oils (EOs) were determined against 10 isolates of *Vibrio parahaemolyticus* from white shrimps *Penaeus vannamei* which were diagnosed with Acute Hepatopancreatic Necrosis Disease (AHPND) or Early Mortality Syndrome (EMS). The objectives of this study are to determine the antimicrobial activities and minimum inhibitory concentration (MIC) of EOs against *V. parahaemolyticus* AHPND pathogen using Kirby-Bauer disc diffusion test and agar dilution method. Five EOs at four different concentrations (100%, 50%, 5% and 2.5% in dilution with 96% ethanol) were tested to determine their antimicrobial activities. Cinnamon, *Cinnamomum zeylanicum* showed the lowest MIC ranging from 0.39-0.78 mg L^{-1} followed by lime, *Citrus aurantiifolia* and lemongrass, *Cymbopogon citratus*, 1.56-3.12 mg L^{-1}, garlic, *Allium sativum,* 3.12-6.25 mg L^{-1} and lemon, *Citrus limon* with value ranging from 6.25-12.50 mg L^{-1}. The MIC of garlic and lemon were at the same level with gentamicin antibiotic (6.25 mg L^{-1}). The qualitative Kirby-Bauer disc test for antimicrobial activities of five EOs showed a significant variation ($P \leq 0.05$) in diameter of inhibition zone (DIZ, mm) against 10 *V. parahaemolyticus* AHPND isolates. Lime EO (100% concentration) showed high antimicrobial activities (Mean, 19.0±2.7), followed by cinnamon (17.4±3.9) and lemon (16.2±3.9). Garlic and lemongrass EOs showed low to moderate antimicrobial activities against *V. parahaemolyticus* with DIZ, Mean, 10.2±2.1 and 9.9±2.9, respectively. This study showed high potential of EOs as an antimicrobial agent in disease control and treatment against AHPND caused by *V. parahaemolyticus* bacteria in shrimp aquaculture.

Keywords: Antimicrobial activities, essential oils, *Vibrio parahaemolyticus*, white shrimp

Abstrak: Antimikrobial aktiviti daripada lima pati minyak tumbuhan (EOs) komersial ditentukan terhadap 10 isolat *Vibrio parahaemolyticus* daripada udang putih *Penaeus vannamei* yang dikenalpasti berpenyakit Acute Hepatopancreas Necrosis Disease (AHPND) atau Early Mortality Syndrome (EMS). Objektif kajian ini adalah untuk menentukan aktiviti antibakterial dan kepekatan minimum merencat (MIC) pertumbuhan bakteria (MIC) daripada EOs terhadap AHPND patogen melalui disk serapan esei Kirby-Bauer dan kaedah pencairan agar. Cinnamon, *Cinnamomum zeylanicum* menunjukkan MIC terendah dengan nilai di antara 0.39-0.78 mg L^{-1} diikuti oleh limau nipis, *Citrus aurantiifolia* dan serai wangi, *Cymbopogon citratus*, 1.56-3.12 mg L^{-1}, bawang putih, *Allium sativum* 3.12-6.25 mg L^{-1} dan lemon, *Citrus limon,* dengan nilai di antara 6.25-12.50 mg L^{-1}. Nilai MIC bagi bawang putih dan lemon EOs adalah setaraf dengan antibiotik gentamicin (6.25 mg L^{-1}). Ujian kualitatif disk serapan esei Kirby-Bauer bagi lima EOs menunjukkan signifikan variasi ($P \leq 0.05$) bagi aktiviti antimikrobial dalam diameter zon rencatan (DIZ, mm) terhadap 10 *V. parahaemolyticus* AHPND isolat. Lime EO (100% kepekatan) menunjukkan aktiviti antimikrobial

yang tinggi (Mean, 19.0±2.7), diikuti oleh cinnamon (17.4±3.9) dan lemon (16.2±3.9). Bawang putih dan serai wangi menunjukkan aktiviti antibakterial dengan zon serapan yang sederhana terhadap 10 *V.parahaemolyticus* dengan DIZ, purata, 10.2±2.1 and 9.9±2.9, mengikut turutan. Kajian ini menunjukkan potensi tinggi EOs sebagai antimikrobial agen di dalam rawatan dan kawalan penyakit AHPND disebabkan oleh *V. parahaemolyticus* bakteria dalam akuakultur ternakan udang.

Introduction

A new emerging shrimp disease known as Acute Hepatopancreatic Necrosis Disease (AHPND) or Early Mortality Syndrome (EMS) has been reported to cause significant economic losses to shrimp farmers worldwide with cases first reported in China (2009), Vietnam (2010), Malaysia (2011) and Thailand (2012) (Lightner *et al.*, 2012; NACA-FAO, 2011, Kua *et al.*, 2016). The disease affects black tiger shrimp (*Penaeus monodon*), Pacific white shrimp (*Penaeus vannamei*) and chinese/oriental white shrimp (*Penaeus chinensis*) characterized by mass mortalities reaching up to 100% in some cases during the first 20 to 30 days of culture (Lightner *et al.*, 2012). When the disease is already present in a farm, strict hygienic practice and biosecurity measures are usually advised to prevent the spreading of disease. Application of probiotics in water or disinfection with chlorine or ozone will eliminate multiple pathogens and maintain a mature microbial community. Feed additive supplement containing essential oil (EO) such as an oregano EO has been applied and reported able to reduce *Vibrio* species population, lower the bacterial counts in hepatopancreas and shrimp muscle (Gracia *et al.*, 2014). *Vibriosis* has been documented in shrimp culture as one of the major infectious diseases worldwide including *V. parahaemolyticus* that are shown to be pathogenic because of the *tdh* and/or *trh* gene and other virulence factors (Zhang *et al.*, 2016). The *Pir*-like toxins known as *ToxA&ToxB* were eventually identified as the cause of AHPND due to *V. parahaemolyticus* infection (Sirikharin *et al.*, 2015). Binary toxins called *ToxA* (12.7 kDa) and *ToxB* (50.1kDa) that resemble binary *PirA&PirB* insecticidal toxins from *Photorhabdus* species in amino acid sequence and in gene arrangement have been characterized as virulent factors that cause AHPND pathology in shrimps (Dong *et al.*, 2017; Leobert *et al.*, 2017; Sirikharin *et al.*, 2015; Han *et al.*, 2015; Lee *et al.*, 2015; Yang *et al.*, 2013).

The EOs are known to possess various biological functions such as anti-inflammatory (Singh and Majumdar, 1999), antibacterial and antifungal (Burt, S.A., 2004; Ouattara *et al.*, 1997), antiviral (Aruoma *et al.*, 1996), antioxidant (Calabrese *et al.*, 1999) and anticancer (Kumar *et al.*, 2004). Cinnamon, lime, lemon, lemongrass or citronella and garlic EO have been widely used in natural therapies since ancient times. The use of medicinal plants or EO extract as prevention or treatment against parasitic or bacterial diseases in fish and shrimp farming have been increasing. Plant extracts such as *piper betel* 'sireh' extract has been effectively used as feed additive for treatment in prevention of fish disease such as *Vibriosis* and Motile Aeromonas Septicaemia (Nik- Haiha *et al.*, 2014; Siti-Hawa *et al.*, 2016). EOs are commercially available, thus, easily accessible to farmers, cost effective, easy to administer, safer and non-toxic (Soares *et al.*, 2016; Soares *et al.*, 2017a).

Various citrus fruits have been reported for its antimicrobial effect inclusive of both gram negative and gram positive bacteria (Owhe-Ureghe *et al.*, 2010). The phenolic compound in these fruits are responsible for the wide spectrum of antimicrobial activity (Dorman and Deans, 2000). Lime crude extract have been reported to be very effective against *V. cholerae* (Jayana *et al.*, 2010). Cinnamon and oregano have been used as growth promoting agent in livestock industry as they are well known for their antimicrobial properties against animal pathogenic bacteria (Alp *et al.*, 2012).

Cinnamon oil is an efficient antibacterial agent against bacteria such as *Escherichia coli*, *Salmonella*, *Staphylococcus* and *Vibrio* spp. (Mith *et al.*, 2014; Zhang *et al.*, 2016; Kaskatepe *et al.*, 2016). Thus, the aim of this study is to qualitative and quantitatively determine the *in vitro* antimicrobial activities of commercial EOs against ten *V. parahaemolyticus* AHPND isolates via disc diffusion test and agar dilution method.

Materials and Methods

Test Isolates

About ten isolates of *V. parahaemolyticus* were obtained from *P. vannamei* white shrimps diagnosed with AHPND occurred in Malaysia from 2011 until 2014. The identification of *V. parahaemolyticus* bacteria was carried out using the conventional method of Gram Stain, oxidase, characteristic growth on Tryptose Citrite Bile Salt (TCBS) agar, sensitivity to Vibriostat agent O/129 (10 and 150 µg/disc), positive oxidative-fermentative sugar test and motile. The isolate was further confirmed by API 20 NE Kit (Bacteria Identification System, Biomérieux, Marcy-l'Etoile, France). Identification using API 20 NE produced 98 to 99.9% similarity to the referred strain for API 20 NE Identification System. The *V. parahaemolyticus* isolate on TCBS agar produced a green colony bacteria and confirmed by PCR to determine AHPND pathogen. A conventional PCR test was performed using a detection kit (IQ2000^{TM}) as confirmatory for *V. parahaemolyticus* AHPND with Toxin 1 and plasmid detection.

Antibacterial susceptibility test

Five EOs were obtained from Sigma Aldrich (St. Louis, USA). The antibacterial activities of cinnamon *Cinnamomum zeylanicum,* garlic *Allium sativum*, lime *Citrus aurantiifolia*, lemon *Citrus limon* and lemon grass *Cymbopogon citratus* EOs were qualitatively determined using Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966). Antibacterial activities of five EOs stock (100%) and three other concentrations were prepared at 50%, 5% (1/20) and 2.5% (1/40) using ethanol (96%) as solvent. Approximately 10 µL of the prepared EO was saturated on a sterile paper disc (6 mm diameter) (Biomérieux, Marcy-l'Etoile, France) placed on the surface of a freshly (24 h) inoculated bacteria on Mueller Hinton Agar added with 1.5% NaCl. Three replicates of disc per test isolate were performed including a negative control disc (saturated with 96% ethanol) and positive control of Oxytetracycline antibiotic (30 µg/disc). Diameter of a clear zone surrounding each disc was marked as diameter of inhibition zone (DIZ) in millimetre after deduction of the disc size (6 mm). The antimicrobial activities measured by the inhibition zone with diameter >16 mm was considered as highly active. Diameter in the range of 12-16 mm were considered moderately active and those < 12 mm were considered as low (Velu *et al.*, 2014).

Bacteria dilution was prepared by taking a few isolates from 24 h fresh culture bacteria on a tryptose soy agar plate incorporated with 1.5% NaCl and mixed into 2 mL normal saline (0.9% NaCl) solution to obtained 0.5 MacFarland concentration containing 10^8 cfu mL^{-1} bacteria colony. About 100 µL (10^6 cfu) bacterial solution was spread evenly across the surfaces of the Mueller Hinton agar plates using a sterile cotton swab to produce a confluent lawn of growth. After the discs were applied, the plates were incubated at 30 °C for 24 h. Diameter of inhibition zone (DIZ, mm) for EOs were expressed as mean of three replicates (n=3) after deduction of the control disc size (6 mm).

Minimum Inhibitory Concentration (MIC)

The MIC was determined using agar dilution method (NCCLS, 2000). Several colonies of each bacterial strain were sampled with a sterile cotton swab, then diluted in 2 mL 0.9% NaCl in order to get a bacterial suspension of 10^8 cfu mL^{-1} for inoculation. A series of two-fold dilutions of each EOs, ranging from 100% concentration (v/v) to 0·03% (v/v), was prepared in Mueller Hinton agar incorporated with 0·5% (v/v) Tween-20. Plates were dried at 35 °C for 30 min prior to spot inoculation with 5 µL of the tested bacteria containing approximately 10^6 cfu per test of EO. Each test was performed in triplicate. Mueller Hinton agar, with 0·5% (v/v) Tween-20 but with no tested EO was used as a positive control. Inoculated plates were incubated at 30 °C for 24 h. The MICs were determined as the lowest concentration of EO inhibiting the visible growth of each bacteria inoculated into the well on Mueller-Hinton agar plate. The presence of one or two colonies was disregard.

Table 1: Preparation of antibiotic (gentamicin) and EOs stock for MIC using agar dilution method with two-fold serial dilution (NCCLS, 2000).

No.	EO stock (mg L^{-1})	vol. dilution (0.5% Tween-20 distilled water) (mL)	EO vol. (mL)	EO (1mL) in 20 mL MHA^+ plate agar (mg L^{-1})
1.	1000	5	5	50
2.	500	5	5	25
3.	250	5	5	12.5
4.	125	5	5	6.25
5.	75	5	5	3.16
6.	32.5	5	5	1.56
7.	16.2	5	5	0.78
8.	8.13	5	5	0.39
9.	4.62	5	5	0.20
10.	2.32	5	5	0.10
11.	1.16	5	5	0.05
12.	0.58	5	5	0.02

Statistical analysis

The mean value and standard deviation of Diameter of Inhibition Zone (DIZ, mm) were calculated from three replicates of test. Analysis of variance ANOVA was performed to determine the significant difference between EOs ($P \leq 0.05$). Statistical analysis was undertaken using Microsoft Windows SPSS v.21 (IBM, USA).

Results and Discussion

V. parahaemolyticus Identification

Selected *V. parahaemolyticus* bacteria were subjected to phenotypic identification. Typical colonies of *V. parahaemolyticus* growth culture are green colour, 2 - 3 mm in size on TCBS, oxidase-positive, Gram-negative, rod curve shaped and motile. Vibrio genus was further identified to species level using the API 20 NE. Biochemical analysis using API 20 NE test results are shown in Table 2.

Ten isolates of *V. parahaemolyticus* were obtained from white shrimp, *P. vannamei* with clinical signs of slow growth, lethargy, soft shells, whitish muscle, empty stomach and midgut, and pale atrophied hepatopancreas that often have black streaks. The AHPND diseased in shrimp was diagnosed with the support from histopathological changes observed in samples such as massive sloughing of hepatopancreatic tubule epithelial cells (Kua *et al*., 2016). The results for PCR analysis using IQ2000^{TM} are shown in Fig. 1. Five isolates were carrying non-virulent AHPND plasmid (no. 2, 4, 6, 8, 12) and only one isolate (no. 13) was positive for AHPND carrying toxin gene. The other 4 isolates were negative (no. 15, 1, 11, 5) for AHPND Toxin 1 or AHPND plasmid detection.

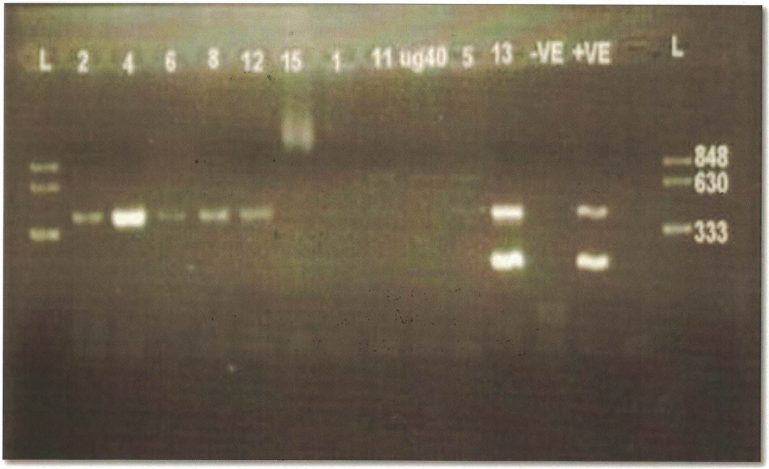

Figure 1: Gel electrophoresis of PCR products showing AHPND Toxin 1 gene sequence detection at 218 bp amplicon and plasmid gene sequence carrying AHPND toxin at 432 bp amplicon in sample number 13. Sample number 2, 4, 6, 8 and 12 were showing non-virulent AHPND plasmid with Toxin 1 deletion.

Table 2: Bacteria identification for *V. parahaemolyticus* using API 20 NE Identification System and other biochemical test.

No.	Test ID	Vp1	Vp2	Vp4	Vp5	Vp6	Vp8	Vp11	Vp12	Vp13	Vp15
1.	Nitrate (NO_3)	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
2.	L-Tryptophane (TRP)	+	+	+	+	+	+	+	+	+	+
3.	D-glucose (GLU)	-/+	-/+	+	-/+	+	+	+	+	+	+
4.	L-arginine (ADH)	-	-	-	-	-	-	-	-	-	-
5.	Urea (URE)	-	-	-	-	-	-/+	-	-	-	-
6.	Esculin (ESC)	-/+	-/+	-	-/+	-	-	-	-	-/+	-
7.	Gelatin (GEL)	+	+	+	+	+	+	+	+	+	+
8.	4-Nitrophenyl-βD-galactopyranoside (PNPG)	+	+	+	+	+	+	+	+	+	+
9.	Assimilation D-glucose (GLU)	-	+	+	-	-	+	+	+	-	+
10.	Assimilation L-arabinose (ARA)	+	+	+	+	+	+	+	+	+	+
11.	Assimilation D-mannose (MNE)	+	+	+	+	-	+	+	+	-	+
12.	Assimilation D-mannitol (MAN)	+	+	+	+	+	+	+	+	+	+
13.	Assimilation N-acetyl-glucosamine (NAG)	-	+	+	-	+	+	+	+	-	+
14.	Assimilation D-maltose (MAL)	+	+	+	+	-	+	+	+	+	+
15.	Assimilation Potassium gluconate (GNT)	+	+	+	+	+	+	+	+	+	+
16.	Assimilation Capric acid (CAP)	-	-	-	-	-	-	-	-	-	-
17.	Assimilation Adipic acid (ADI)	-	-	-	-	-/+	-	-	-	-/+	+
18.	Assimilation Malic acid (MLT)	+	+	+	+	+	+	+	+	+	+
19.	Assimilation Trisodium citrate (CIT)	-	-	-/+	-	-	-/+	-	-	-	-
20.	Assimilation Phenylacetic acid (PAC)	-	-	-	-	-	-	-	-	-	-
21.	Oxidase (OX)	+	+	+	+	+	+	+	+	+	+
22.	Vibriostat O129 (150 & 10 µg)	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S
23.	Color growth on TCBS	green	green	green	green	green	green	green	green	green	green

Table 3: Antimicrobial activities for five EOs with mean Diameter of Inhibition Zone (DIZ, mm) against 10 strains of *V. parahaemolyticus*.

EOs (%)	Cinnamon	Lime	Lemon	Garlic	Lemon grass	Skewness (symmetry)	Kurtosis (tail distribution)
	Diameter of inhibition zone in mm (Range, Mean±SD)						
i) 100	8 - 23 (17.4±3.9)	11 - 26 (19.0±2.7)	7 - 23 (16.2±3.9)	7 - 14 (10.2±2.1)	4 - 14 (9.9±2.9)	0.442	-0.937
ii) 50	7 - 21 (14.5±2.4)	8 - 19 (14.3±2.7)	3 – 14 (10.4±2.4)	4 – 12 (6.8±2.3)	4 – 13 (6.6±2.9)	0.251	-1.029
iii) 5	6 – 10 (7.8±2.5)	6 – 9 (7.4±2.0)	5 – 9 (6.9±2.7)	4 – 8 (5.9±2.6)	5 – 9 (6.3±2.8)	0.441	-0.702
iv) 2.5	4 – 8 (5.9±2.3)	4 – 7 (5.3±1.9)	4 – 7 (5.5±2.5)	3 – 7 (5.1±2.6)	3 – 7 (5.2±3.1)	0.582	-0.455

Five EOs at four different concentrations (100%, 50%, 5% and 2.5% in dilution with 96% ethanol) were tested to determine their antimicrobial activities. Lime, cinnamon and lemon were showing high antimicrobial activities against 10 *V. parahaemolyticus* isolates. Antimicrobial activities of lime and cinnamon at 100% or 50% concentrations were moderate (DIZ ≥ 12 - 16) to high (DIZ > 16) and further dilution with absolute ethanol (5% and 2.5%) showed a reduction in DIZ as the concentration were reduced (Table 3). Overall, lime showed high antimicrobial activities at 100% concentration with DIZ ranging from 11 - 26 mm (mean, 19.0±2.7), cinnamon, 8 - 23 (17.4±3.9), lemon, 7 - 23 (16.2±3.9), garlic, 4 - 14 (10.2±2.1) and lemongrass, 4 - 14 (9.9±2.9). The DIZ of EOs at four different concentrations (100%, 50%, 5% and 2.5%) against 10 strains of *V. parahaemolyticus* are shown in Table 3 (Mean; DIZ, mm). ANOVA One-way analysis showed that antimicrobial activities of EOs at 100% ($F_{4,44} = 12.898$, $P = 0.000$; $P \leq 0.05$) and 50% ($F_{4,44} = 17.513$, $P = 0.000$; $P \leq 0.05$) are significantly different as shown in Fig. 2 (100%) and Fig. 3 (50%).

Lime EO (100% concentration) showed high DIZ against 90% of *V. parahaemolyticus* isolates (DIZ, 16 - 26 mm) followed by cinnamon (DIZ: 16 - 23 mm) in 70% of test isolates and lemon (DIZ: 16 - 23 mm) in 60% of test isolates (Fig. 2). The antimicrobial activities of garlic and lemongrass EOs are moderate (DIZ; 12 - 16 mm) against 30% and 20% of *V. parahaemolyticus* isolates whereas low inhibition zone (DIZ; < 12 mm) were recorded for other isolates (70 - 80 %) (Fig. 2). Post Hoc Test for multiple comparison using Tukey showed that lime and lemon, cinnamon and lemon were groups as EOs with highest antimicrobial activities at 100% and 50% concentrations ($P \leq 0.05$) whereas garlic and lemongrass were categorized as moderate (Fig. 2 & 3).

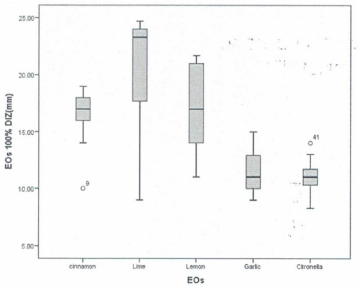

Figure 2: Boxplot showing antimicrobial activities of EOs at 100% concentration

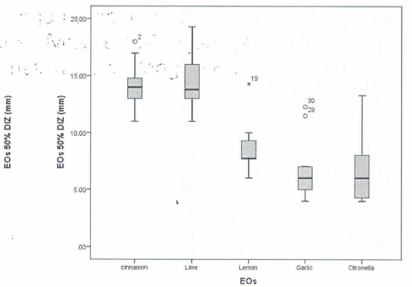

Figure 3: Boxplot showing antimicrobial activities EOs at 50% concentration

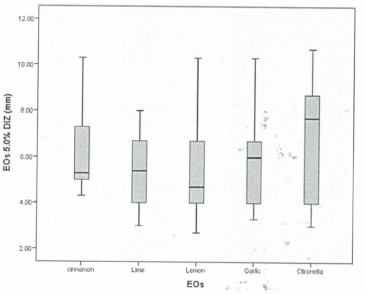

Figure 4: Boxplot showing antimicrobial activities EOs at 5% concentration

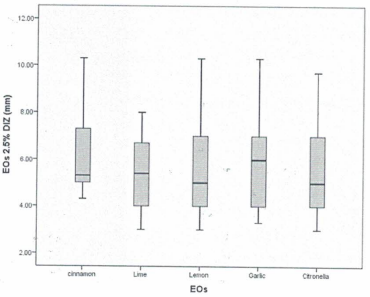

Figure 5: Boxplot showing antimicrobial activities EOs at 2.5% concentration

Statistical analysis using ANOVA One-way showed no significant different ($P \geq 0.05$) between EOs at 5% ($F_{4,44} = 0.483$, $P = 0.748$; $P \geq 0.05$) and 2.5% concentration. ($F_{4,44} = 0.185$; $P = 0.945$, $P \geq 0.05$). High variation in DIZ value were recorded between 10 *V. parahaemolyticus* isolates (Fig. 4 and 5). Eight *V. parahaemolyticus* isolates are sensitive toward oxytetracycline (30 µg/disc) antibiotic (S: DIZ >19) with two intermediate (I: DIZ, 14 - 18). The zone of inhibition (DIZ) and Mean of EOs at 5% concentration for cinnamon, 6 - 10 (7.8±2.5), lime, 6 - 9 (7.4±2.0), lemon, 5 - 9 (6.9±2.7), garlic, 4 - 8 (5.9±2.6) and lemongrass, 4 - 9 (6.3±3.1) are not significantly different ($P \geq 0.05$) from DIZ for the respective EOs at 2.5% concentrations; cinnamon, 4 - 8 (5.9±2.3), lime, 4 - 7 (5.3±1.9), lemon, 4 - 7 (5.5±2.3), garlic, 3 - 7 (5.1±2.6) and lemongrass, 3 - 7 (5.2±2.8). Results are shown in Table 3 (Range, Mean±SD) and (Fig. 4 and 5). The zone of inhibition for EO lemon and lime against *V. parahaemolyticus* isolates are shown in Fig. 6 and Fig. 7.

Minimum Inhibitory Concentration (MIC)

The lowest MIC with value ranging from 0.39 - 0.78 mg L^{-1} were observed with cinnamon EO followed by lime (1.56 - 3.12 mg L^{-1}), lemongrass (1.56 - 3.12 mg L^{-1}), garlic (3.12 - 6.25 mg L^{-1}) and lemon (6.25 - 12.5 mg L^{-1}). The sensitivity of cinnamon, lime and lemongrass against *V. parahaemolyticus* AHPND strains were high and comparable with gentamicin antibiotic (6.25 mg L^{-1}). The results are shown in Table 4.

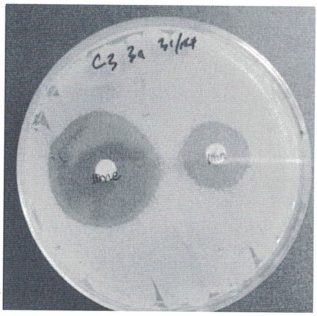

Figure 6: DIZ for lime and lemon (100% concentration) against Vp4.

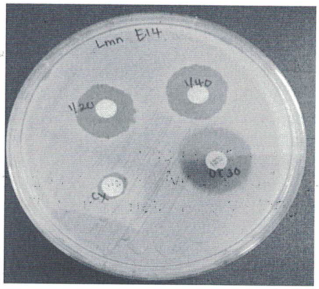

Figure 7: DIZ for lemon EO at 5% and 2.5% concentrations against Vp11 isolate in comparison with negative control and antibiotic Oxytetracycline (OTC 30 µg).

Table 4: The minimum inhibitory concentration (MIC) of EOs against *V. parahaemolyticus*.

No.	Bacteria Id	MIC (mg L^{-1})					
		cinnamon	garlic	lime	lemon	lemon grass	gentamicin
1.	Vp1	0.78	6.25	1.56	6.25	3.12	6.25
2.	Vp2	0.78	6.25	1.56	6.25	3.12	6.25
3.	Vp4	0.78	6.25	3.12	6.25	3.12	6.25
4.	Vp5	0.78	6.25	1.56	6.25	3.12	6.25
5.	Vp6	0.78	6.25	3.12	6.25	3.12	6.25
6.	Vp8	0.78	3.12	1.56	12.5	1.56	6.25
7.	Vp11	0.39	3.12	1.56	12.5	1.56	6.25
8.	Vp12	0.78	3.12	1.56	12.5	1.56	6.25
9.	Vp13	0.78	6.25	3.12	6.25	3.12	6.25
10.	Vp15	0.78	3.12	1.56	12.5	1.56	6.25

A new source of antimicrobial agent is needed to replace the growing concern of chemical and antibiotic usage in aquaculture industry. Overuse and abuse of antibiotic in feed as growth promoter and for treatment have caused the resistance and failure in treatment. This study showed that moderate to high antimicrobial activities were recorded from cinnamon and lime EOs against 10 *V.*

antimicrobial agent in feed could be further explored in disease management and control program to benefit shrimp culture or fish farming industry. High sensitivity of *V. parahaemolyticus* AHPND local strains to cinnamon and lime EOs showed their potential use as feed additive in an effort to reduce the *Vibrio* spp. population in shrimp's hepatopancreas and gut. Lime and cinnamon EOs were able to inhibit *V. parahemolyticus* growth at 50 and 100% concentrations with the lowest MIC ranging from 0.39 - 1.32 mg L^{-1}. Consequently, chances of bacterial colonization to the gut epithelium in association with AHPND disease occurrence is reduced (Gracia *et al.*, 2014).

Moderate antimicrobial activities of lemon (60%), garlic (30%) and lemongrass (20%) EOs against *V. parahaemolyticus* test isolates showed their potential as bacteriostatic agent at lower concentration. Thus, lime, cinnamon, lemon, garlic and lemongrass EOs are beneficial as antimicrobial agent or functional diet (additive) in pelleted feed to reduce microbial population in gastrointerstinal system as part of disease control and prevention treatment against *V. parahaemolyticus* shrimp pathogen. Active compound of hydrocarbons (lemonine, a and b-pinene, c-terpinene), and monoterpenes such as a-terpineol, sabinene, q-cymene, neryl and geranyl acetates are reported to be major components contributing to high antimicrobial activities in lemon (Fisher and Phillips, 2008). Lime consist of flavonoids components which lead to its antimicrobial, antioxidant and anticancer properties whereas the main organic acid component, citric acid is believed to have contributed to the prominent antimicrobial results (Jayana *et al.*, 2010). In addition, the EOs possess important volatile compounds with diverse bioactivities including antioxidant properties which are important as food preservative, growth promoter (amino acids and vitamins) as well as to improve flavour and aroma.

Conclusion

This study showed moderate to high antimicrobial activities of lime, cinnamon and lemon against ten *V. parahaemolyticus* isolates with DIZ mean value ranging from 12-26 mm in diameter. Moderate antimicrobial activities of lemon (60%), garlic (30%) and lemongrass (20%) EOs showed their potential as bacteriostatic agent against *V. parahaemolyticus* AHPND pathogen. Quantitative analysis using agar dilution method showed that cinnamon EO exhibit the lowest MIC ranging 0.39 - 0.78 mg L^{-1} followed by lime and lemon grass with MIC of 1.56 - 3.12 mg L^{-1}. The MIC for EOs garlic and lemon varies between test isolates ranging from 3.12 - 12.5 mg L^{-1} with sensitivity comparable with antibiotic Gentamicin (6.25 mg L^{-1}). This study showed the potential use of EOs cinnamon, lime, lemon, garlic and lemongrass as antimicrobial agent for AHPND treatment at higher concentrations (100% and 50%) or as feed additive/functional diet at lower concentration (2.5% and 5%) in shrimp farming.

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Challenge Study of Single and Mixed AHPND's Bacterial Isolates Against Pacific Whiteleg Shrimp (*Penaeus vannamei*)

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Abstract: A challenge study was done to determine the pathogenicity and LD_{50} of single and mixed bacterial isolates of AHPND towards Whiteleg Shrimp. Isolate *Vibrio parahaemolyticus* (E14) from previous AHPND's positive case and isolate *V. alginolyticus* (A1) from previous epidemiological study were tested against Whiteleg Shrimp in a 24-hour experiment. Results obtained from the study showed that the LD_{50} for single isolate challenge test (E14) was 5.83×10^9 CFU ml^{-1} while for mixed isolates challenge test (E14 & A1) was 5.38×10^9CFU ml^{-1}. Detection using IQ2000 ems2 kit did not showed positive sample band although cumulative mortality result showed otherwise. Mixed isolates challenge test showed better LD_{50} result compared with single isolate test suggesting multiple Vibrio roles in triggering AHPND outbreak in Whiteleg Shrimp.

Keywords: LD_{50}, single isolate, mixed isolates, pathogenicity, AHPND

INTRODUCTION

Early mortality syndrome is a new disease that has been detected at shrimp farms in Asia. It appears within 30 days of stocking and causes symptoms that include lethargy; soft, darkened shells and mottling of the carapace [1]. EMS/AHPNS initially surfaced in 2009. By 2010 outbreaks had become serious. In 2011, farms in China (Hainan, Guangdong, Fujian and Guangxi) suffered almost 80 percent losses. In Thailand, shrimp production for 2013 is predicted to be down 30 percent from last year due to EMS. Production on some farms in eastern parts of the country has been cut by 60 percent [2]. Since EMS was first reported in China in 2009, it has spread to Vietnam, Malaysia and Thailand, and now causes annual losses more than U.S. $1 billion [3]. Both black tiger shrimp, *Penaeus monodon*, and Pacific white shrimp, *Litopenaeus vannamei*, are affected by the disease [1]. The disease is caused by a pathogenic strain of *Vibrio parahaemolyticus* [4]. In this study, we try to determine the pathogenicity and LD_{50} of single and mixed isolates (*V. parahaemolyticus*) towards Whiteleg Shrimp as multiple *Vibrio* species were commonly found in AHPND's outbreak in shrimp pond. Isolate E14 was chosen because it dwells in the same group as pathogenic isolate *V. parahaemolyticus* (3HP) from Thailand and isolates A1 because it was isolated from AHPND positive epidemiological study.

MATERIALS AND METHODS

Bacterial isolates

Isolates used in this study (E14 and A1) were obtained from previous positive AHPND mortality case and epidemiological study. Isolates were preserved in Brain Heart Infusion Broth with 10% added glycerol and stored in -80^{0}C freezer before being subcultured onto Tryptic Soy Agar with added 1.5% NaCl prior to use.

Test animals
Animals used in this study were of age DOC29. Animal were fed 2-3 times daily with pelleted feed and acclimatized for 3 weeks prior to experiment.

Preparation of bacterial suspension
Beads containing bacterial isolate were streaked onto Tryptic Soy Agar with added 1.5% NaCl and were incubated overnight at 30^0C. About 10 colonies of bacteria were inoculated in TSB+ and incubated overnight at 30^0C in shaking incubator to achieve a bacterial density of 10^9 CFU ml-1.

Immersion challenge test
Immersion procedure was carried out by immersing 10 shrimp for 5 minutes in a plastic aquarium containing solution of 500ml bacterial suspension in TSB+. Shrimps were transferred back in their respective aquaria after immersion.

Experiment 1: Immersion study with individual bacterial isolate:
Twenty 100L glass tanks were used in this experiment representing 4 replicates of 4 bacterial concentrations (10^9, 10^8, 10^7 and 10^6) and negative control (normal saline without bacteria). A total of 10 individual shrimps were immersed for 5 minutes in different bacterial concentrations with adequate aeration and immediately transferred back to their respective aquarium after immersion.

Experiment 2 : Immersion study with mixed bacterial isolates:
Twenty 100L glass tanks were used in this experiment representing 4 replicates of 4 bacterial concentrations (10^9, 10^8, $10^7$and 10^6) and negative control (normal saline without bacteria). A total of 10 individual shrimps were immersed for 5 minutes in different bacterial concentrations with adequate aeration and immediately transferred back to their respective aquarium after immersion.

Observation
Shrimp were observed and mortality was counted after 16 and 24 hours post-experiment. A total of 2 individual shrimps were sampled from each concentration post experiment and subjected to Polymerase Chain Reaction (PCR) using IQ2000 ems2 kit (GeneReach) to confirm the infectious agent in the experiment.

RESULTS AND DISCUSSION

Results obtained from the study showed that the LD_{50} for single isolate challenge test (E14) was 5.83 x 10^9 CFU ml^{-1} while for mixed isolates challenge test (E14 & A1) was 5.38 x 10^8 CFU ml^{-1} **(Figure 1)**. LD_{50} for mixed isolates challenge test was lower compared to of single isolate challenge test; thus indicating the possibilities of multiple *Vibrio* species role in AHPND outbreak. Polymerase Chain Reaction detection of the samples using IQ2000 ems2 kit did not showed positive sample band **(Figure 2 & 3)** although mortality was observed in the experiment. Our suggestion was to do histopathological analysis of the samples in this study to confirm the pathogenic effect in this experiment.

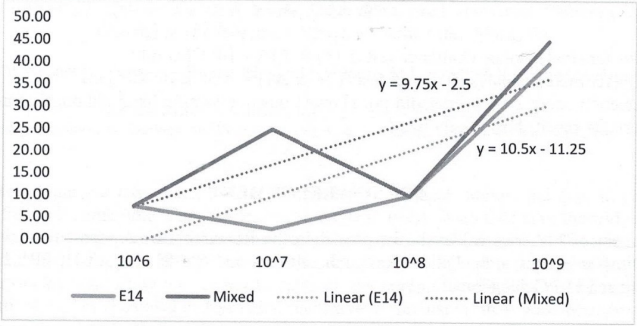
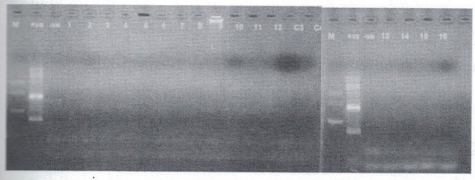
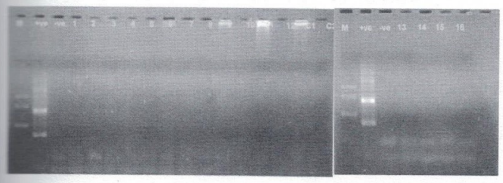


Figure 1: Cumulative mortality of shrimp vs. bacterial concentration in challenge study



Lane 1 = marker, Lane 2 = positive control,
Lane 3 = negative control
1 = conc. 10^9 R1, 2 = conc. 10^8 R1
3 = conc. 10^7 R1, 4 = conc. 10^6 R1
5 = conc. 10^9 R2, 6 = conc. 10^8 R2
7 = conc. 10^7 R2, 8 = conc. 10^6 R2
9 = conc. 10^9 R3, 10 = conc. 10^8 R3
C3 – C4 = Control, 11 = conc. 10^7 R3
12 = conc. 10^6 R3, 13 = conc. 10^9 R4
14 = conc. 10^8 R4, 15 = conc. 10^7 R4
16 = conc. 10^6 R4

Figure 2: IQ2000 PCR result from single isolate challenge test



Lane 1 = marker, Lane 2 = positive control,
Lane 3 = negative control
1 = conc. 10^9 R1, 2 = conc. 10^8 R1
3 = conc. 10^7 R1, 4 = conc. 10^6 R1
5 = conc. 10^9 R2, 6 = conc. 10^8 R2
7 = conc. 10^7 R2, 8 = conc. 10^6 R2
9 = conc. 10^9 R3, 10 = conc. 10^8 R3
C1 – C2 = Control, 11 = conc. 10^7 R3
12 = conc. 10^6 R3, 13 = conc. 10^9 R4
14 = conc. 10^8 R4, 15 = conc. 10^7 R4
16 = conc. 10^6 R4

Figure 3: IQ2000 PCR result from mixed isolates challenge test

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CONCLUSION

- LD_{50} for single isolate challenge test (E14) = 5.83×10^9 CFU ml^{-1}
- LD_{50} for mixed isolates challenge test (E14 & A1) = 5.38×10^9 CFU ml^{-1}
- Detection using AP4 primer did not showed positive sample band although cumulative mortality result showed otherwise.

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RESEARCH Update

Updates on EMS/AHPND Research at NaFisH

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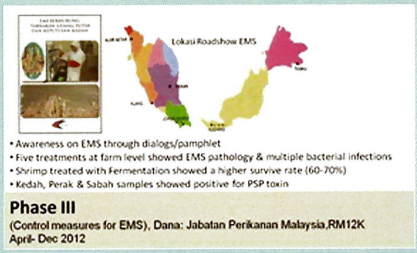
In the middle of 2011, Fisheries Research Institute through National Fish Health Research Division (NaFisH) has received two reports from shrimp operators in Perak on the occurrences of white feaces and slow death of cultured white-leg shrimp. Subsequently, in September 2011, DOF of Malaysia was informed by the Malaysian Shrimp Farmers Association of high mortalities at alarming rates in most of the shrimp farms throughout Peninsular Malaysia. Immediate action was taken to determine the possible cause or aetiology of the outbreak.

Phase 1 and 2

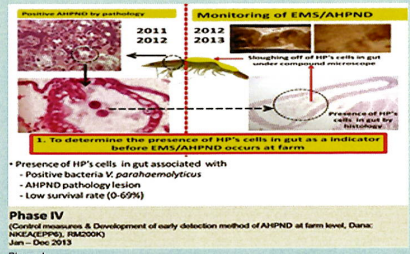
Following the initial investigation, a series of studies were conducted in five phases which covered confirmation of EMS, finding the associated factors with EMS occurrence and control measure through R&D. The high mortality was later confirmed as early mortality syndrome (EMS) or known as acute hepatopancreatic necrosis disease (AHPND) in 2011. The awareness programmes were then included in phase III.



Phase 3

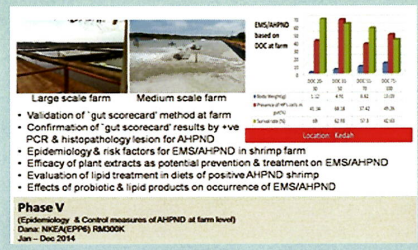
Due to the acute and high mortalities caused by EMS/AHPND, some form of control measures of EMS/AHPND were taken by farmers at the affected sites but the effectiveness of the control measure were uncertain. This led to the need for more effective control measures by R&D such as in-situ early detection at the farm, prevention and treatment of EMS/AHPND.

In Phase IV, development of early indicator such as the presence of hepatopancreas cells in the gut before the occurrence of EMS/AHPND was carried out.



Phase 4

Concurrently, several products were tested at laboratory while a few products were also introduced at the site. Apart from that, validation of the `gut scorecard' along with the confirmation of EMS/AHPND by PCR and histology was implemented in Phase V. Epidemiology and studies on the potential remedy for EMS/AHPND were also included in Phase



Phase 5

V. Following the phase V results, a seminar and dialogue session with stakeholders were conducted on 24th October 2015. During the National seminar on Aquaculture, under shrimp section,the results from phase V were presented.

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