THE *ASIARESIST* PROJECT: A STUDY OF ANTIMICROBIAL RESISTANCE ASSOCIATED WITH ASIAN AQUACULTURAL ENVIRONMENTS

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Abstract: Stimulated by concerns relating to the consequences of illegal use of antibiotics in aquaculture, the EU-funded ASIARESIST project brought together three European and three Southeast Asian (SEA) partners to develop knowledge and tools to assist identification and monitoring of antibiotic resistance in aquaculture in Southeast Asia. Following development of appropriate standard operating procedures (SOPs), a sampling of water, sediment and cultured organisms on representative farms in the three SEA countries was conducted using a screen for bacterial resistance to chloramphenicol (CHL). Resistance to CHL and to other antibiotics was found in all sites studied on all sampling occasions. DNA fingerprinting reduced the primary collection of 724 resistant bacterial isolates to 558 by omitting replicate isolates on the basis of unique DNA banding patterns. The majority of CHL-resistant isolates were found to belong to six bacterial families, namely the Enterobacteriaceae (63%), the Pseudomonadaceae (12%), the Moraxellaceae (11%), the Bacillaceae (6%), the Micrococcaceae (3%) and the Aeromonadaceae (2%). The remaining isolates belonged to other families, namely the Alcaligenaceae, Comamonadaceae, Enterococcaceae, Flavobacteriaceae and Sphingobacteriaceae. Overall, eight genera, Escherichia (39%), Pseudomonas (12%), Acinetobacter (11%), Klebsiella (7%), Bacillus (5%), Staphylococcus (3%), Citrobacter (3%) and Enterobacter (3%) dominated the total strain collection. In most cases CHL resistance levels reached MIC values of 512 ppm or more, and was accompanied by resistance to multiple antibiotics. The majority of the Gram-negative isolates tested carried cat *genes, with* cat2 *(74%) being more common than* cat1 *(26%). No isolates were found to carry the* cat3 *gene. Of those isolates not displaying evidence of a* cat *gene, the majority of those tested showed biochemical evidence of CHL acetyl transferase (CAT) activity. In* vitro *conjugation studies revealed transfer of CHL resistance and other resistance phenotypes from aquaculture isolates to a laboratory strain of* E.coli. *The methods, tools and bacterial collection developed by ASIARESIST will be made generally available for research and survey/monitoring use.*

Keywords - **Aquaculture – Southeast Asia – chloramphenicol – antimicrobial resistance – heterotrophic bacteria – ASIARESIST – bacterial diversity – web-based database**

I. INTRODUCTION

Antibiotics are widely used in aquaculture for the treatment and prevention of bacterial infections in cultured species. In some regions, farmers of aquatic species would be unable to maintain a viable enterprise without the use of antibiotics. While alternative strategies such as vaccination, probiotic administration and farming of genetically resistant stock are being developed and tested, the use of antibiotics remains the most cost effective means to combat bacterial diseases for many farmers. Antibiotics are also widely used in clinical medicine and in terrestrial agriculture. However, their use in aquaculture poses specific dosage problems. As aquatic species are farmed at high densities, individual dosing is impractical. The alternatives are either addition to feeds or to the water. In both cases this results in the introduction of antibiotics at varying concentrations in the aquatic environment in the proximity of farms. This situation is likely to trigger the selection and dissemination of resistance to these (and other) antibiotics in the autochthonous bacterial population of aquaculture sites. As a consequence, antibiotics may no longer be effective in controlling disease, and the only alternative maybe to abandon or fallow the site. Furthermore, there is a public perception that the rise of antibiotic resistance in aquaculture environments may also have an impact on the human population, including farmers and consumers. The vast majority of bacteria that occur in aquaculture sites are harmless to humans or other animals, including the cultured species. However, certain groups of indigenous bacteria occurring at high densities may play a role as reservoir organisms of antibiotic resistance triggered by the selective pressure of antibiotic use and the horizontal transfer of resistance genes. Of particular concern is the potential of human pathogens, in interacting with the aquaculture environment, to acquire new resistance traits through genetic exchange with resistant subpopulations of the autochthonous microbiota.

As in most areas of food production, antibiotic usage in aquaculture is generally legitimate and carefully managed. However, there are indications that the worldwide ban of several antibiotics may have led to the illegal use of specific

antibiotic compounds. In the period 2000-2002 residues of the broad-spectrum antibiotic chloramphenicol (CHL), which is banned for use in the aquaculture-practising countries of Asia and Southeast Asia (SEA), were detected in farmed shrimp. As highly publicised in the international media, the European Community reacted to this development by banning the import of several Asian aquaculture products.

Chloramphenicol is a particularly important antibiotic that is generally reserved for the treatment of serious infections of the central nervous system and eye, and some highly contagious and potentially epidemic diseases such as typhoid. In these recent cases the foods were banned because they contained residues of CHL. Low levels of CHL in food products not only generate toxicity concerns, but could lead to the development of antibiotic resistance in bacteria that are carried by the consuming public. In addition, the presence of CHL residues in aquaculture products indicates use of CHL at farm level, with the consequent risk that antibiotic resistance may develop and spread throughout the farming environment. Once clinical pathogens acquire new resistance traits there is a potential risk to those people working on farms and those handling the farm products as they are prepared for sale. Also, if the resistance arises in pathogens of the species that is being farmed the antibiotic will cease to be of any value to the farmer, whether it is due to legal or illegal use.

The aim of the European Union (EU)-funded ASIARESIST project (contract no. ICA4-CT-2001-10028) is to determine the extent to which antibiotic resistance may occur in SEA aquaculture environments, to assess the potential of antibiotic resistance gene transfer between bacteria, to raise awareness of the problem, and to develop protocols and tools for farmers and disease control agencies that can be used to monitor antibiotic resistance and prevent its spread into the human food chain. To this end, in December 2001, a unique collaboration between three research groups in Europe (Institute of Aquaculture, University of Stirling, UK; Laboratory of Microbiology, Ghent University, Belgium; RILAB srl, Genova, Italy) and three in SEA (College of Aquaculture and Fisheries, University of Can Tho, Vietnam; Faculty of Veterinary Medicine, University Putra Malaysia, Malaysia; Department of Fisheries, Thailand) was established with the support of the EU. The objective, over a period of three years, was to develop and disseminate expertise and tools that could be applied to detect and monitor levels of antibiotic resistance in bacteria in aquaculture environments and products of SEA. The beneficiaries would be both the aquaculture-practicing countries, and their customers in the EU and elsewhere.

II. METHODOLOGY AND RESULTS

The project comprises seven inter-related work packages (WP) (Fig. 1): harmonisation and standardisation of sampling methodology and antimicrobial susceptibility testing; sample collection and bacterial isolation; taxonomic characterisation of strain collection; antimicrobial susceptibility testing; genotyping of isolates; resistance gene detection and horizontal transfer studies; data curation, analysis, and information dissemination.
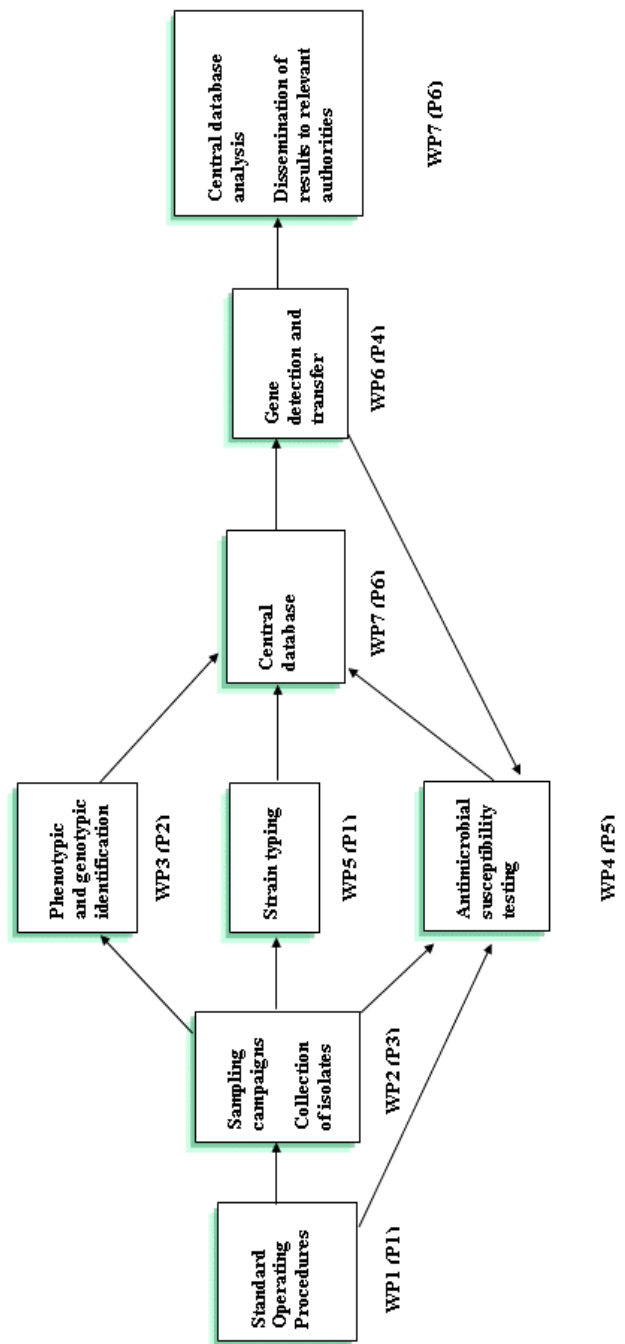

Fig.1. Project work plan

WP 1: Evaluation, harmonisation and standardisation of methodologies;

The first WP defined standard operating procedures (SOPs) for the project sampling protocols and antimicrobial susceptibility testing of bacterial isolates from aquaculture environments.

The first SOP document, ASIARESIST-SAMPLE, detailed the processing of water, sediment, and cultured species samples recovered from the selected aquaculture sites. As a consequence of field trials led by the Thai project partner, a sampling pipe was re-designed to improve the sampling of pond sediments.

The ASIARESIST project targets a specific subset of bacteria from the aquaculture environment, namely CHL-resistant heterotrophic bacteria. A breakpoint concentration of 35 ppm CHL was chosen, following a preliminary survey of the CHL-resistant population, to be included in the Iso-sensitest agar (ISA) isolation medium. The sampling strategy was designed to provide a project strain collection representative of the CHL-resistant bacterial population capable of aerobic growth at 28°C on ISA.

The SOP document ASIARESIST-PRES described the maintenance and long-term storage of the project strain collection. A key output for the ASIARESIST project was the formulation of another SOP, ASIARESIST-ABR, for the antibiotic susceptibility testing of bacterial isolates from the SEA aquaculture environment by the disc diffusion (DD) assay. Similarly, a SOP for determining the minimum inhibitory concentration (MIC) of CHL, ASIARESIST-MIC1, was developed. The suitability of the above SOPs was evaluated by testing a panel of 10 control strains by the project partners.

WP 2: Sample collection, bacterial isolation and preservation

In order to establish a heterogeneous collection of heterotrophic CHL-resistant bacterial isolates from various aquaculture environments, and to initiate the project-specific database, sampling campaigns were conducted in Thailand, Malaysia and Vietnam based on the SOPs for sampling, sample processing and preservation.

A wide range of farms in different locations and using different farming systems was initially screened for CHL-resistant isolates at 35 ppm. Twelve aquaculture sites were finally selected for the project sampling campaign. These comprised four sampling sites in Thailand (Chachengsao, Chanthaburi, Patumthani and Supunburi), three in Malaysia (Banting, Ipoh and Batu Gajah) and five in Vietnam (Con Au, Thot Not, Omon, Vinh Long and Tan Phu Thanh). The sites either practised monoculture or integrated culture systems. Sites were also chosen to be representative of the diversity of the economically important species cultured in the target countries. The cultured species included *Cyprinus carpio*, *Pangasius hypophthalmus*, *Clarias macrocephalus* X *Clarias gariepinus*, *Lates calcarifer*, *Oreochomis niloticus*, *Aristichthys nobilis*, *Penaeus monodon*, *Osphronemuss goramy* and *Trichogaster pectoralis*. At each farm, one or more ponds were identified and selected for sample collection, and sampling was performed four times in a period of three months. The sampling intervals ranged from two to three weeks. Three sample types, water, sediment and farmed species (organism), were collected on each sampling occasion. Samples were plated on ISA medium supplemented with 35 ppm CHL to isolate CHL-resistant heterotrophs. In addition, samples were plated on ISA medium without CHL to collect isolates susceptible to CHL as potential recipient strains for future horizontal transfer experiments.

At the end of the sampling campaign, a total of 724 CHL-resistant isolates and 102 'susceptible' isolates were collected and cryopreserved. The heterotrophic bacterial isolates isolated from aquaculture sites in each country were stored in the country of origin and in the central strain collection maintained by Ghent University, Belgium.

WP3: Taxonomic characterisation of the strain collection

Studies focusing on the selection and dissemination of antibiotic resistance in aquaculture environments have often failed to fully describe the diversity of the bacterial hosts carrying the genetic determinants of antibiotic resistance. ASIARESIST conducted a polyphasic identification program for the taxonomic characterisation of CHL-resistant isolates originating from aquaculture environments and cultured species in Malaysia, Thailand and Vietnam. First, the set of isolates was dereplicated using rep-PCR DNA fingerprinting which reduced the original set of 724 isolates to 558 on the basis of unique DNA banding patterns. As an initial identification approach, all isolates were subjected to gas-liquid chromatographic analysis of cellular fatty acid methyl esters (FAMEs). Comparison of the unknown FAME profiles with the heterotrophic identification database TSBA of the Sherlock Identification System (Microbial ID Inc., Newark, DE, USA) allowed the classification of all 558 investigated isolates to at least bacterial family level. Distribution analysis of the identification results revealed that the majority of the CHL-resistant isolates belonged to six bacterial families, namely the Enterobacteriaceae (63%), the Pseudomonadaceae (12%), the Moraxellaceae (11%), the Bacillaceae (6%), the Micrococcaceae (3%) and the Aeromonadaceae (2%). The remaining isolates belonged to other families, namely the Alcaligenaceae, the Comamonadaceae, the Enterococcaceae, the Flavobacteriaceae and the Sphingobacteriaceae.

In order to obtain further in-depth identification of the isolates to genus or species level, additional techniques were adopted as a follow-up to FAME analysis, including phenotypic characterisation using the commercial systems API 20E, API 50E and API 32 ID STAPH (bioMérieux, France), partial or complete 16S rDNA sequence analysis, protein profiling and amplified ribosomal DNA restriction analysis (ARDRA) fingerprinting. Based on the current set of identification data, it is clear that members of the Enterobacteriaceae (notably *Escherichia coli*) largely dominated the collection of isolates across the three sampled countries. Overall, 8 genera, namely *Escherichia* (39%), *Pseudomonas* (12%), *Acinetobacter* (11%), *Klebsiella* (7%), *Bacillus* (5%), *Staphylococcus* (3%), *Citrobacter* (3%) and *Enterobacter* (3%) dominated the CHL-resistant strain collection and are represented by at least one isolate in all

three country subcollections. Genera of which representatives were found in two of the three sampling countries included *Aeromonas* (2%) and *Comamonas* (1%). Although the relative frequencies with which they occur differed significantly, some of these groups may thus be regarded as possible indicator organisms for monitoring CHL resistance in aquaculture environments. At the species level, enterobacterial CHL resistant isolates were mainly found to belong to *E. coli*. In addition, a portion of the isolates belonged to primary or opportunistic human pathogens such as *Acinetobacter baumannii* and *Klebsiella pneumoniae*. Enterobacterial isolates also dominated across the various sampling types (i.e. water, sediment, and cultured species) although it was clear that the enterobacteria were more prevalent in the cultured species samples by comparison with sediment or water sample types. In contrast, strains of *Acinetobacter* and *Bacillus* rarely occurred in the cultured species samples. In the case of *Bacillus*, the majority of the isolates were found in sediment.

WP4: Antimicrobial susceptibility testing of bacterial isolates from Southeast Asian aquaculture

Antimicrobial susceptibility testing of aquaculture-associated bacteria using the disc diffusion method indicated susceptibility of the challenged organism to the tested antibiotic by a clear zone of inhibited growth around impregnated paper discs. The diameter of the resulting zone was considered proportional to the degree of susceptibility and allowed the categorization of tested organisms as susceptible (S), intermediate (I), or resistant (R) to a particular antibiotic according to international guidelines.

It was clear that the heterotrophic bacterial population in aquaculture sources comprised bacteria that were both susceptible and resistant to CHL. Overall, the three country subcollections of CHL-resistant bacteria were similar. The majority of CHL-resistant isolates (82.6%) showed a MIC =>512 ppm. They also exhibited multi-resistance to CHL and ampicillin (AM), tetracycline (TE), nitrofurantoin (F/M) and trimethoprim/sulfamethoxazole (SXT) at various frequencies, but were relatively susceptible to norfloxacin (only 28% being resistant to this antibiotic). The most common profiles were of resistance to 3 and 4 antibiotics (14% and 15% of tested isolates, respectively), the commonest resistance profiles being CHL-AM-TE-SXT and CHL-AM-TE-SXT-F/M. Resistance to CHL alone was rare (3%). Moreover, there was a tendency for CHL-resistant isolates with higher MIC values to be resistant to greater numbers of antibiotics than those with lower MICs. Similarly, bacterial isolates from integrated farms showed resistance to more antibiotics than those obtained from monoculture farms (99% confidence interval).

WP5: Genotyping of isolates

The strain typing aimed to establish the epidemiological relationships among the CHL-resistant isolates by the application of molecular typing techniques. The information obtained was used to compare the genetic relationships between the isolates and to select representative clonal lineages for subsequent gene detection studies.

A repetitive (rep)-PCR typing method, directed by the repetitive primer (GTG)$_5$, was initially applied to screen the project isolates. The resulting DNA profiles were numerically analysed to compare the individual isolates. Strain variation within the three countries varied from 60-80%. Related isolates that were shown to yield identical (GTG)$_5$-PCR profiles from the same samples were designated "replicates" of the same strain and removed from the project collection for subsequent analysis purposes.

Macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) was employed as an independent typing method to confirm the genetic relationships inferred by (GTG)$_5$-PCR among the project isolates. Isolates that appeared indistinguishable by (GTG)$_5$-PCR or that gave rise to ambiguous results were selected for macrorestriction analysis. The protocol involved digesting the bacterial chromosome with infrequently cutting restriction enzymes and resolving the large DNA fragments using a specialised form of gel electrophoresis. The method was able to confirm the presence of replicates (identical strains from the same sample type) and indicate potential strain associations occurring between different sites, ponds, time points and sample types.

WP6: Resistance gene detection and horizontal transfer

This WP aimed to characterize the genes responsible for mediating CHL resistance among the project strain collection and to assess the potential for horizontal transfer of these genes.

Enzymatic acetylation of CHL, mediated by the CHL acetyl transferase (CAT), is one of the most common mechanisms of CHL resistance. A multiplex PCR assay directed at three *cat* genes (*cat*1-3) known to occur in Gram-negative bacteria was applied to the project isolates. The majority (63%) of the Gram-negative isolates tested carried *cat* genes, with *cat*2 (47%) being more common than the *cat*1 (16%) gene. No isolates were found to carry the *cat*3 gene.

The isolates found to carry neither *cat*1 nor *cat*2 genes, mainly belonged to *Acinetobacter*, *Pseudomonas*, and among the Gram-positive genera, to *Bacillus* and *Staphylococcus*. A selection of these isolates (n=97) was subjected to a biochemical CAT assay. The majority of the isolates were found to possess CAT activity, with a minor proportion, equally represented by Gram-negative and Gram-positive organisms, showing no detectable CAT activity (n=24). The results indicated that both CAT and non-enzymatic mechanisms of CHL resistance may be involved.

The ability of the resistance genes to transfer between bacteria was assessed using a plate-based conjugation assay and the proven laboratory recipient strain *E. coli* HB101. CHL-resistant project isolates found to be susceptible to streptomycin were combined with the highly streptomycin-

resistant recipient HB101. A double selective medium containing 35 ppm CHL and 800 ppm streptomycin was used to select for potential transconjugants. In 113 donor/recipient combination tests, a total of 32 transconjugants were confirmed. Transconjugants typically originated from donors belonging to the Enterobacteriaceae family and involved the transfer of sulphonamide and tetracycline resistance as well as CHL resistance. Plasmid profiling of the transconjugants revealed that large plasmids > 100 kb) were commonly associated with the transfer of resistance to *E. coli* HB101.

WP7: Data analysis, management and dissemination

Internet is the fastest growing tool and arguably the most popular method of accessing public domain information. The World Wide Web (WWW) has become an interactive environment where server, client, various devices and users are able to interact at different levels, sharing software and business services. As a result, an on-line project central database (DB) was developed by RILAB srl (www.rilab.it) and placed on the project website in the first months of the project. The project website was structured in two different websites, one public (www.medinfo.dist.unige.it/asiaresist) and accessible to all users, and one private, accessible only to the project partners, which housed the on-line project results DB. The public project website aims to disseminate the main objectives of ASIARESIST and, on completion of the project, to make available the project outputs.

The on-line ASIARESIST DB exploits the use of developing information and communication technologies. This inter-disciplinary and inter-sectorial research DB of project results is structured in four sections:

1. User information
2. Site-Sample-Strain description; Strain identification and confirmation; CAT and strain typing results
3. Antibiogram and MIC results
4. Search engine through the DB.

At present, the DB recorded information on 623 confirmed strains, of which 143 originated from Malaysia, 251 from Thailand and 229 from Vietnam. Of these strains, 558 were CHL-resistant and 65 were CHL susceptible.

The identification, antibiotic susceptibility (antibiogram and MIC), CAT gene and typing results were entered into the DB and are available using the DB search facility.

One of major objectives of the ASIARESIST project includes the broad dissemination of the research results and main conclusions. Following the project's completion in May 2005, the ASIARESIST DB will be opened to the public and the data collected as well as the data analysis and conclusions will be made available thorough a public portal.

III. DISCUSSION AND CONCLUSION

The AIARESIST project has created and refined a number of SOPs and data management systems to assist those concerned with assessing the occurrence and extent of resistance to CHL and other antibiotics in aquaculture environments. When these were applied during surveys of sediment, water and farmed organisms in a range of aquaculture systems in Malaysia, Thailand and Vietnam, resistance to CHL and to a number of other antibiotics was found at all sampling sites on every sampling occasion. More than 500 unique CHL resistant bacterial isolates, and a smaller collection of susceptible isolates, have been preserved and archived in Ghent University and in the countries of origin.

With respect to the diversity of bacteria harbouring resistance, six bacterial families and eight genera dominated the resistant strain collection, with the Enterobacteriaceae being the largest group and *E. coli* the commonest species. Significantly, potential human pathogens were among the antibiotic-resistant isolates in all three countries where studies were conducted.

The majority of CHL resistant isolates exhibited MIC values of 512 ppm CHL or more, and, with only a small number of exceptions, CHL resistance was accompanied by resistance to other antibiotics. Indeed, the majority of isolates was resistant to three or more antibiotics.

Studies of resistance revealed that it was commonly associated with CAT activity, and in most cases this could be related to the presence of *cat*1 or *cat*2 genes. Moreover, the capacity of some aquaculture isolates to transfer multiple resistance phenotypes to *E. coli* was demonstrated.

In summary, a refined suite of tools and standardised methods for detection and monitoring of antibiotic resistance in aquaculture environments are now in place. To date they have revealed widespread antibiotic resistance in aquaculture in all three Southeast Asian countries where they have been applied. There is no reason to suppose that other countries in the region will be different in this respect, but studies are urgently required to assess the situation.

Control of antibiotic resistance is clearly in the interests of all those involved at the various points in the food chain, from farmers to consumers. Hopefully the advances made by the ASIARESIST project will become the basis for a significant and concerted effort to achieve an acceptable and sustainable level of control. To that end it is the policy of ASIARESIST to make full details of methods generally accessible through the project WWW site in the near future. Representative bacterial strains of the ASIARESIST collection will be made available to researchers through the BCCM[TM]/LMG Bacteria Collection (Laboratory of Microbiology) of Ghent University.