Summary of final report

OBJECTIVES

Use of antibiotics in the aquaculture environments of South East Asia (SEA) poses a risk that resistance to antibiotics will develop in environmental organisms, in the endogenous bacterial population of farmed species, and in their pathogens. Antibiotic resistant bacteria constitute a direct threat to farmers, consumers and other forms of livestock through potential transfer of resistance to human and animal pathogens, and threaten the viability of export markets.

The project therefore aimed to:

- Obtain a preliminary indication of the extent of antibiotic resistance in SEA aquaculture;
- Identify the resistance genes involved;
- Assess the potential for transfer of antibiotic resistance from the aquaculture environment to possible human pathogens;
- Identify critical control points (CCP) at which SEA fish farmers can apply monitoring systems to prevent or eliminate antibiotic resistance;
- Disseminate control protocols among farming and disease control communities in SEA.

In this context the objectives were as follows:

General

1. Assessment of the extent of antibiotic resistance in the aquaculture environment in South East Asia (SEA)
2. Assessment of the potential for transfer of antibiotic resistance from the aquaculture environment to the broad public environment
3. Identification of critical control points (CCP) where south East Asian fish farmers can apply monitoring systems to prevent or eliminate antibiotic resistance

Specific

1. In work package 1 to formulate a set of standard operating procedures designed for project activities.
2. In work package 2 to establish a collection of oxytetracycline resistant heterotrophic bacteria from diverse Asian geographical and aquaculture backgrounds.
3. In work package 3 to provide descriptive and taxonomic data for each antibiotic-resistant bacterial isolate for inclusion in the project specific database.
4. In work package 4 to provide quantitative data on the antimicrobial susceptibility of each bacterial isolate.
5. In work package 5 to produce plasmid profiles for each antibiotic-resistant bacterial isolate.
6. In work package 6 to describe the distribution of resistance genes among a sub-set of multi-resistant plasmid-harbouring isolates.
7. In work package 6 to assess the transferability of resistance between aquatic and human bacterial isolates.
8. In work package 7 to establish a global database to collect data in a standard format.
9. In work package 7 to produce guidelines for monitoring transferable antimicrobial resistance at aquaculture sites and web-accessible strategies for the implementation of hazard analysis and critical control point (HACCP) ecosystem management.

ACTIVITIES

In broad terms the activities were:

- Development of Standard Operating Procedures (SOPs) for use in SEA aquaculture systems for bacterial sampling, isolation and preservation, for antibiotic susceptibility

testing and determination of the extent of antibiotic resistance, for resistance plasmid isolation and for bacterial conjugation assays.

- Provision of a taxonomic description of new bacterial taxa isolated from SEA aquaculture environments.
- Characterisation of resistance to antimicrobials in bacterial populations from different compartments of SEA aquaculture ecosystems.
- Determination of the distribution of resistance genes among a sub-set of antibiotic resistant plasmid-harbouring bacterial isolates.
- Assessment of transferability of resistance from the aquaculture ecosystem.
- Development of a core public database dealing with monitoring of antibiotics in aquaculture environments.
- Dissemination of new tools methods and knowledge.

This project investigated the extent and nature of antibiotic resistance in ecosystems exploited for aquaculture purposes in SE Asia. The approach was to apply standardised, quality controlled (QC) protocols in representative aquaculture sites in Vietnam, Malaysia and Thailand that covered a range of environmental conditions, management practices and antibiotic usage histories.

The project comprised seven interrelated tasks:

1. Standardisation of sampling methodologies for isolation of antibiotic resistant and non-resistant control bacteria.
2. Isolation of chloramphenicol-resistant and non-resistant control bacteria.
3. Taxonomic characterisation of the bacterial strain collection.
4. Qualitative and quantitative assessment of the antimicrobial susceptibility of bacterial isolates.
5. Genotyping of bacterial isolates.
6. Resistance gene detection and assessment of potential for horizontal transfer.
7. Data analysis, management and dissemination.

Activities and methodologies were as follows:

Standardisation of sampling methodology

Objective: To define standard operating procedures (SOP) for a.) sampling aquaculture environments for chloramphenicol-resistant bacterial isolates and b.) for handling and storage of isolates.

Methods: The methods followed those laid out by the Working Group on Antimicrobial Agent Susceptibility Testing of Bacteria Associated with Fish Diseases (http://www.nuigalway.ie/mic/eusus/index.html.). The project consortium targeted a specific subset of bacteria from the aquaculture environment i.e. the culturable fraction of the heterotrophic chloramphenicol (CHL)-resistant population.

Sample collection, bacterial isolation and preservation

Objectives:

1. To assemble a heterogeneous collection of heterotrophic antibiotic resistant bacterial isolates from a range of SE Asian aquaculture environments based on a series of standard operating procedures
2. To initiate the project-specific database by the incorporation of sampling and isolation data for each partner involved

Methods: Following the relevant SOPs sampling of sites was undertaken in Malaysia, Thailand and Vietnam where species representative of the majority of the aquaculture industries in the three countries were being farmed. These were:

Vietnam (Mekong delta):	black tiger shrimp, pangasius catfish and common carp
Malaysia:	Black tiger shrimp, red tilapia and freshwater giant prawn (Macrobrachium rosenbergii).

Thailand:	shrimp, sea bass, and tilapia (integrated with poultry rearing)

On each farm site samples were taken from water, sediments, and from the intestine of farmed fish/shellfish. The sampling process was undertaken four times at each site.

Taxonomic characterisation of the strain collection.

Objectives:

1. To assess the taxonomic diversity of a heterogeneous collection of antibiotic-resistant freshwater and marine isolates originating from SE Asian aquacultural environments.
2. To provide identification data to be included in the project-specific integrated database.

Methods: Clonal relationships were studied by repetitive chromosomal element-PCR analysis (rep-PCR) and PFGE and "non-duplicate" strains based on repPCR results were subjected to gas-liquid chromatographic analysis of cellular fatty acid methyl esters (FAMEs). When necessary, this was followed up with second choice techniques for species identifications of specific bacterial groups.

Descriptive and taxonomic data were systematically compiled for each isolate and entered into the central project database system for further processing into an integrated data format.

Antimicrobial susceptibility testing

Objectives:

1. To harmonise and evaluate susceptibility testing using internal and external quality controls
2. To test the susceptibility of all isolates to antimicrobials used in aquaculture, agriculture and human medicine.
3. To provide quantitative data on the level of antimicrobial resistance in bacteria isolated from aquaculture products.

Methods: In order to ensure standardisation of methods across the project SOPs were developed for antibiotic susceptibility testing by the disc diffusion method and for determination of minimal inhibitory concentration of antibiotic by the broth macrodilution method.

Genotyping of isolates

Objectives:

1. To study the presence of plasmids in antibiotic-resistant isolates originating from Asian aquaculture environments
2. To establish epidemiological relationships among a selection of antibiotic multi-resistant bacteria
3. To determine whether antibiotic resistance at geographical diverse aquatic environments is due to the introduction and spread of antibiotic-resistant clones, or to the spread of resistant genes.
4. To select a panel of donor and recipient strains for studies on gene transfer

Methods: Plasmid extraction and genotypic methods were evaluated for their suitability in characterising bacteria encountered within the aquaculture environment. A repetitive (rep)-PCR, directed by the $(GTG)_5$ primer was chosen as a typing method to initially screen the project isolates to check for strain identity and so reduce the numbers of isolates for subsequent analysis. DNA templates were prepared from boiled extracts from broth cultures. A standardised PCR and gel electrophoresis protocol using a commercial PCR master mix and the $(GTG)_5$ primer was developed. Dendrograms were created for each country by GelCompar analysis to compare the PCR profiles of the individual isolates.

Macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) was then applied to confirm the genetic relationships inferred by rep-PCR among the project isolates.

A plasmid extraction method was optimised to be suitable for profiling plasmids from aquaculture-associated bacteria. The method was designed to be capable of detecting large molecular weight (> 50 kb) plasmids previously encountered in plasmid-mediated antibiotic resistance and avoid the use of hazardous solvents.

Resistance gene detection and horizontal transfer

Objectives:

1. To compose and validate a panel of specific antibiotic resistance gene probes
2. To analyze the occurrence of antibiotic (tetracycline) resistance genes in a subset of the isolated strains defined in WP 4.
3. To study the potential for interspecific and intergeneric genetic exchange between aquatic and human bacterial isolates.
4. To investigate direction and type of transfer occurring.

Methods: A multiplex PCR assay for the detection of CHL acetyl transferase genes (*cat*1-3) was applied to the strain collection. Those project isolates shown not to possess *cat* genes were tested for CAT activity in a biochemical assay.

Potential project donor isolates were screened for susceptibility to streptomycin, rifampicin, nalidixic acid and ampicillin by disc diffusion. *E. coli* isolate HB101 STR^R was chosen as the recipient strain for the conjugation assay. A conjugation assay based on a plate method was adopted for the study.

A SOP was developed for the extraction of plasmids from aquaculture-associated bacteria. The method was found to be suitable as a screening method for detecting large plasmids (> 100 kb) commonly found within the Enterobacteriaceae and avoided the use of commercial purification columns and hazardous solvents.

Data analysis, management and dissemination

Objectives:

1. To define the general requirements for project specific database
2. To set up a general protocol and a global data base to collect data in a standard format.
3. To establish an internet based platform for restricted internal discussions between partners.
4. Disseminate project results to farmers in the form of workshops and informative leaflets

Methods: A centralised project database was developed in which all partners could record data arising from their studies. Data interchange was facilitated by the use of a standard communication protocol and by the setting up of common user friendly interfaces, in order to optimise the efficiency of data management.

RESULTS ACHIEVED

Evaluation; harmonisation and standardisation of sampling methodology

SOPs were developed as follows:

SOP-ASIARESIST-SAMPLE. The SOP specified that each of the three SEA partners were to conduct sampling campaigns in their respective countries. These were to involve three sites where three different aquaculture species are farmed (fish and shrimp species). One pond was to be chosen on each site for water and sediment sampling in three locations within the pond. The farmed species were also sampled (gut contents), and the three location samples for each sample type (water, sediment and species) were pooled. The sampling was to be conducted on four occasions covering the production cycle. Following careful study a breakpoint for sampling for the collection of CHL resistant isolates was set at 35 ppm, and embodied in the sampling SOP.

SOP-ASIARESIST-PRES. The purpose of this SOP was to describe the procedure for long term storage of bacterial cultures. Organisms were to be stored at low temperatures utilising a mechanical technique that results in minimal disturbance of the material. Considerable effort was expended in ensuring accuracy and consistency in the laboratories involved in handling bacteria

that involved distribution of standard strains by Partner 2 to Partners 1, 3, 4, and 5. These were recovered from transportation media, cryopreserved, recovered and returned to the distributing Partner for confirmation of their identity and freedom from contamination. This exercise was conducted on two occasions.

SOP-ASIARESIST-ABR. The purpose of this SOP was to describe the procedure for testing the antimicrobial susceptibility of bacterial isolates using disc diffusion methodology (i.e. antibiogram determination). The antibiotics chosen for the disc diffusion assays were chloramphenicol, tetracycline, ampicillin, nitrofurantoin, norfloxacin, trimethoprim/sulfamethoxazole 1:19.

Sample collection, bacterial isolation and preservation.

The sampling campaigns required sampling of pond sediments as well as pond water and the cultured species. A new sediment sampling tool was developed for this purpose.

Isolates were made in each of three SEA countries and taken further in the project were as follows:

Country	Isolates	Taken forward to taxonomy studies
Vietnam	300 (250 C + 50 N)	215 (211 C + 4 N)
Thailand	360 (320 C + 40 N)	223 (215 C + 8 N)
Malaysia	166 (154 C + 12 N)	143 (132 C + 11 N)
TOTAL	**826 (724 C + 102 N)**	**573 (558 C + 15 N)**

AAHRI, Partner 5; UPM, Partner 4; CTU, Partner 3.
C, chloramphenicol-resistant isolates (35 ppm); N, chloramphenicol-susceptible isolates

Taxonomic characterisation of strain collection.

Taxonomic diversity of CHL-resistant isolates at the genus level was found to be as follows:

Genus	Thailand	Vietnam	Malaysia	N (%)
Acinetobacter	18	16	28	**62 (11%)**
Aeromonas	1	10		**11 (2%)**
Alcaligenes			1	**1**
Bacillus	24	2	5	**31 (5%)**
Citrobacter	9	8	1	**18 (3%)**
Comamonas		3	3	**6**
Edwardsiella			1	**1**
Enterobacter	2	2	12	**16 (3%)**
Enterococcus	2			**2**
Escherichia	67	120	33	**220 (39%)**
Klebsiella	9	29	4	**42 (7%)**
Morganella	3			**3**
Myroides	1			**1**
Pantoea		2		**2**
Proteus	15			**15 (3%)**
Providencia	6			**6**
Pseudomonas	37	6	24	**67 (12%)**
Salmonella	1	1	1	**3**
Serratia			2	**2**
Sphingobacterium			5	**5**
Staphylococcus	12	5	1	**18 (3%)**
Subtotal (genus ID)	**207**	**204**	**121**	**532 (95%)**
No genus ID	8	7	11	**26 (5%)**
TOTAL	**215**	**211**	**132**	**558**

In many cases identification was taken to species level.

Antimicrobial susceptibility testing

Overall the three country sub-collections of CHL-resistant bacteria were similar. The majority of CHL-resistant isolates (82.6%) showed an 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).

Genotyping of isolates

Rep-PCR and macrorestriction analysis were the methods adopted for genotyping of isolates. Based on the combined typing results, it was estimated that that there were 480 unique types within the strain collection. Following de-replication, the strain variation present within the strain collection was estimated to be 90%. The strain diversity was similar among isolates made in Vietnam and Thailand (88% and 87% respectively). A higher level of strain variation (98%) was noted in the isolates originating from Malaysia where sampling was more limited in scale.

Antibiotic resistance gene detection.

A multiplex PCR assay for the detection of *cat*1-3 genes was applied to the strain collection. The majority of the resistant isolates were found to carry *cat* genes, with *cat*2 being more common than the *cat*1 gene. A proportion of the isolates carried no *cat*1 or *cat*2 genes, mainly representing *Acinetobacter* sp, *Pseudomonas* sp, *Aeromonas* sp and Gram-positive genera.

Among the isolates that were found not to possess *cat*1 or *cat*2 genes, CAT activity was confirmed in the majority (78 %) using a biochemical assay. The group of isolates found not to exhibit any CAT activity under the assay conditions that were applied included members of the *Bacillus* sp, *Staphylococcus* sp, *E. coli*, *Pseudomonas* sp and undesignated *Enterobacteriaceae*. A decreased susceptibility or resistance to florfenicol (FLO; a fluorinated analogue of CHL) was observed by the disc diffusion method in Gram-negative isolates shown not to possess CAT activity. This tended to coincide with the presence of the *cml*A gene or *flo*R gene being detectable by PCR, both examples of efflux-mediated resistance. In contrast, the Gram-positive isolates shown not to possess CAT activity were found to be relatively susceptible to FLO.

In order to assess the prevalence of FLO resistance among the strain collection, a breakpoint determination was carried out at 35 ppm FLO against representatives of the unique strain types defined in WP5. The majority of isolates (67 %) were found to be susceptible to FLO, however, a significant number of project isolates (n= 132) exhibited resistance to FLO in addition to CHL. Resistance to FLO was a universal characteristic of pseudomonads as well as to a proportion of *Acinetobacter* sp and *Enterobacteriaceae* isolates tested.

Gene transfer studies.

For gene transfer studies, donors were derived from the strain collection and the recipient was *E.coli* HB101. Of the donor/recipient combinations tested, a number of transconjugants were confirmed with transfer frequencies ranging from 10^{-4} – 10^{-10}/donor cell. Transconjugants typically originated from donors belonging to the family *Enterobacteriaceae* and involved the transfer of sulphonamide and tetracycline resistance, as well as resistance to CHL.

Project isolates susceptible to CHL and CHL^R isolates V001-V096 were screened for the presence of plasmid DNA. The majority of isolates, regardless of the resistance profile, were found to harbour plasmid DNA often as multiple plasmids and/or including high molecular weight

plasmids. Plasmid profiling of 31 transconjugants revealed that large plasmids (> 100 kb) were commonly associated with the transfer of resistance to the recipient.

Data analysis, management and dissemination

A website was created (http://www.medinfo.dist.unige.it/asiaresist/) for public access to information about the project and its outcomes. A project database for data management was established with web-based access. This web-based structure also contained a continuous discussion area to improve idea and information interchanges between Partners.

In the final stages of the project an International Workshop was organised in Chiang Mai, Thailand, 24th-25th February, 2005, for the purpose of disseminating the project outputs. Following the workshop all abstracts and full papers were organised as an electronic workshop proceedings in .pdf format, according to the Adobe Acrobat Catalogue utility. The workshop Proceedings (ISBN n° 88-901344-3-7) were placed on the public project web site for free download and a CD-ROM copy was sent to all workshop participants.

The project and its outputs were presented to the World Aquaculture Society meeting in 2005.

CONCLUSIONS

- Resistance to CHL is readily detected in aquaculture in all three SEA countries studied.
- Resistance is generally to 512ppm CHL or more.
- Most CHL-resistant isolates are also resistant to at least 3 or 4 other antibiotics. [Resistance to CHL alone is infrequent.]
- The *Enterobacteriaceae* are the dominant resistant group, and *E. coli* the dominant resistant species.
- Some resistant isolates belong to species with clinical relevance.
- Most resistance to CHL is mediated by CAT.
- Most CAT activity is associated with the presence of *cat*1 or *cat*2 genes.
- Resistance to CHL can be transferable from aquaculture isolates to a laboratory strain of *E. coli*.

However, there remains:

- a need to examine the possibility of transfer of antibiotic resistance from aquaculture isolates to potential human pathogens.
- an urgent need to assess the antibiotic resistance situation in aquaculture in other SEA countries.

The most significant outputs of the project are:

- SOPs for
    - sampling of aquaculture environments and farmed species
    - preservation of isolates
    - antibiotic susceptibility testing
    - detection and transfer of antibiotic resistance genes
- An improved sediment sampling tool.
- A comprehensive collection of taxonomically-defined antibiotic-resistant bacterial strains from SEA aquaculture environments.
- CHL MIC data on heterotrophs from aquaculture environments in SEA countries.
- Information on the role of known antibiotic resistance genes in SEA aquaculture.
- Evidence of the potential for antibiotic resistance gene transfer from aquaculture isolates to a laboratory strain of *E. coli*.
- A web-based management system for recording and analysis of antibiotic resistance and strain data.

The most significant benefits arising from this project are:

- Greater awareness of the antimicrobial resistance problem in SE Asian aquaculture resulting from a degree of definition of the problem. This arises from a clear demonstration that

antimicrobial resistance, involving multi-drug resistance at high levels in many cases, is readily encountered in SE Asian aquaculture. This in turn may help ensure that decision makers in the EU and SEA will be able to confront the problem constructively and effectively.

- The availability of effective standardised methods and tools for sampling and processing of samples from aquaculture environments for assessment of antimicrobial resistance, and web-based resources for data management.
- A very valuable scientific resource in the form of a comprehensive collection of antibiotic-resistant bacterial isolates from SEA aquaculture.
- New and potentially productive links between European and SE Asian scientists and research institutions in the aquaculture, bacteriology and information science fields.

From a scientific standpoint, ASIARESIST has been successful. The project can also claim a degree of success in terms of dissemination. In terms of collaboration, both within and between regions, ASIARESIST is a good example of what can be achieved

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