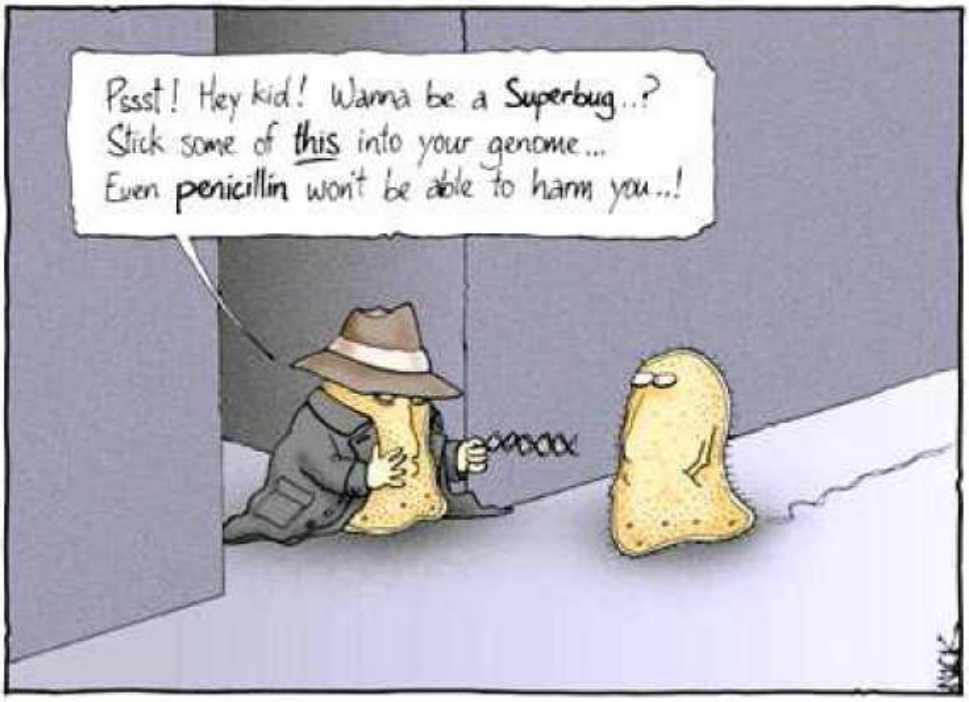
CENTRAL VETERINARY INSTITUTE
WAGENINGEN UR

Pilotstudy:

"Antimicrobial resistance in ornamental fish"



It was on a short-cut through the hospital kitchens that Albert was first approached by a member of the Antibiotic Resistance.

Cartoon by Nick Kim

Research internship

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Pilot study:

"Antimicrobial resistance in ornamental fish"

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Summary

This pilotstudy is performed to investigate the presence and characteristics of antimicrobial resistance in ornamental fish. We focused on the presence of cephalosporin and quinolone resistance. 27 Isolates, selected out of 237 strains isolated in the past five years, where identified by classical and 16SrDNA typing. Antimicrobial disk susceptibility testing was performed using oxytetracylcline, cefotaxim, oxolinic acid and ampicilin, according to the CLSI protocol. These tests where used to select samples for ESBL confirmation testing and MIC determination. These selected samples were also screened using a micro-array and for the presence of mutations in the *gyrA* and *parC* region of the QRDR and by pcr for the presence of specific PMQR's (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, and *aac (6') 1b*- cr). We found point mutations in the gyrA of *Aeromonas* and *Citrobacter* at locations corresponding with data found in recent literature. We also demonstrated the presence of a *qnrB* in the *Citrobacter freundii.* Overall, we found no real ESBL's, but found transmissible quinolone resistance genes in *Citrobacter*. Taken the number of samples, this study indicates a broad presence of antimicrobial resistance in ornamental fish. Therefore, it is concluded that active surveillance of resistance genes of concern for public health like ESBL's and plasmid mediated quinolone resistance genes should be conducted routinely. Also the free purchase by the public, of antimicrobials for ornamental fish, legalized by the five gram regulation should be restricted.

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Introduction

Antimicrobial drugs

History

In 1928 Alexander Fleming discovered penicillin. The sulfonamides where the first effective antibiotics introduced in 1937. In the late 1930's the first antimicrobial resistance patterns where found. In 1940, several years before the launch of penicillin as an antibiotic, the first penicillinases had already been identified. (Abraham and Chain 1940). With today's knowledge, we can explain this phenomenon by the existence of the natural pool of antimicrobial resistance genes. This pool can be exploited for selection in the bacterial population (D'Costa, McGrann et al. 2006). It is of great concern that we will go back to the period were there where no antibiotics available, if the number of resistant bacteria continues to increase (figure 1).

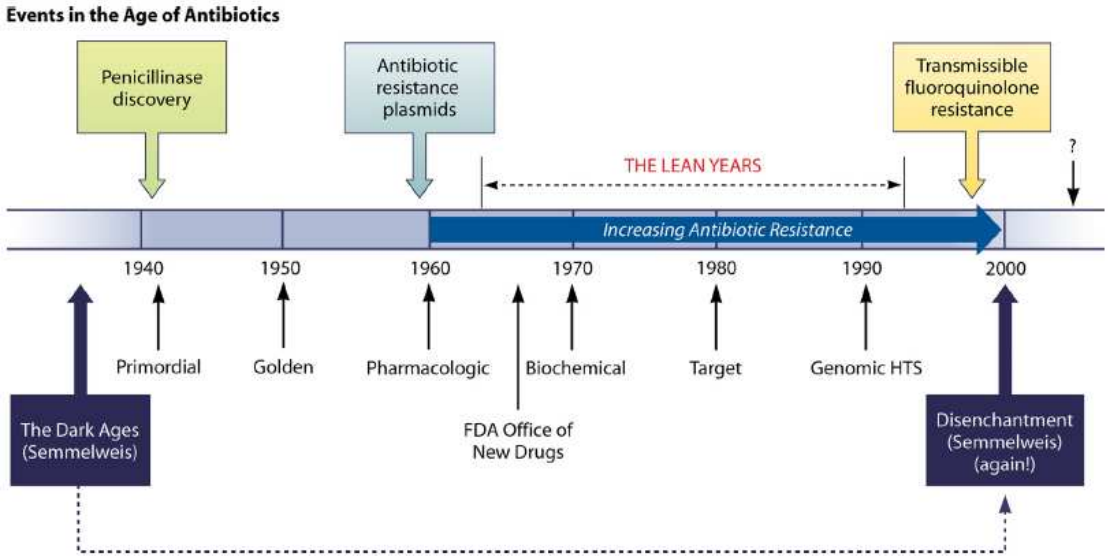


Figure 1 (Davies and Davies 2010)
The course of an era before antibiotics, through a period of little resistance towards a period where the fear of no effective antibiotics is growing. Resulting in the same effects as in the era of no antibiotics.

Antimicrobial agents are used for:

- Growth promotion*/prophylaxis/therapy in farm animals
- Therapy in humans
- Growth promotion*/prophylaxis/therapy in aquaculture
- Therapy/prophylaxis in companion animals
- As cleaning agents

* prohibited in the Netherlands, often applied in the U.S.

Mechanism of antimicrobial effect

Antimicrobials have several ways to inhibit antimicrobial growth or kill bacteria, for the patient to recover from infection. Antimicrobials can inhibit the cell wall synthesis, influence bacterial metabolism, inhibit protein synthesis or damage/inhibit bacterial DNA.

Antimicrobial resistance in general

Despite a restriction in use of antibiotics, the problem of resistance will continue to exist. The mechanisms of bacterial resistance have spread globally over the years (Davies and Davies 2010). It seems that the fastest way to get rid of resistant bacteria is to outnumber them with sensitive bacteria (Levy and Marshall 2004). Treatments with antimicrobial drugs create an inevitable selection on resistant bacteria. When resistant, bacteria survive and multiply, to create even more resistant bacteria. Due to the exchange of resistance genes, non-pathogenic bacteria can become host of potential dangerous resistance genes. When non-pathogenic bacteria exchange these genes to pathogenic bacteria, (multi) resistant bacteria can develop (Bogaard 2000). Animal contribution to the resistance problems in humans are small, yet not insignificant (Levy and Marshall 2004). A large part of the exchange of resistance genes takes place in the gastro-intestinal tract (Levy and Marshall 2004; Davies and Davies 2010). This is a potential risk in animals since a large amount of animals are treated with antibiotics orally, creating a great reservoir for selection in the GI-tract. After excretion, the potential resistant bacteria end up in the environment and near food products of animal origin. These food products are an important source of human infection. Therefore excretion in the environment poses not only a potential treat to animals, but also for humans (Bogaard 2000). Wastewater contains the same potential treat, because of the low concentrations of antimicrobials in the water, selection can occur easily. Subsequently, there is a good opportunity for bacteria to exchange resistance genes among zoonotic, animal, human and commensal bacteria. (Bogaard 2000).
The human population is getting older on average, numbers of vulnerable patients are increasing, as is the number of surgical operations. This causes an increase in immunosuppressed patients, therefore the number of people depended on health care and antibiotic treatments. These sociomedical developments cause an increase in use of antibiotics, which favors the emergence of antibacterial resistance (Chopra, Schofield et al. 2008).

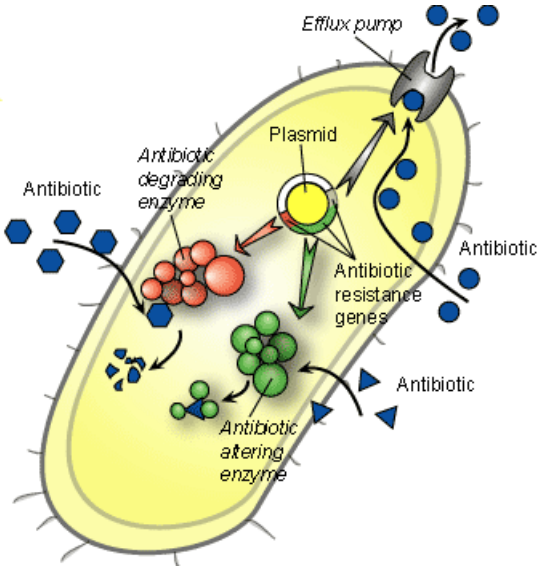
Forms of resistance

Bacteria can have several different ways in which they can be resistant. In principal two mechanisms of resistance can be determined:
Intrinsic resistance
Extrinsic resistance
Intrinsic resistance means the bacterium has a natural form of resistance that is caused by its own (internal) properties. Extrinsic resistance means the bacteria acquired resistance properties, other than its own natural resistance.

Mechanisms of antimicrobial resistance:

Bacteria can develop several different mechanisms to become resistant against one or more antimicrobials:

- Chemical modification (e.g. aminoglycosides, fenicols)
- Active efflux out of the bacterial cell (e.g. tetracyclines)
- Modification of the binding site (e.g. penicillin, macrolides)
- Enzymatic inactivation of the antibiotic (e.g. beta-lactams)



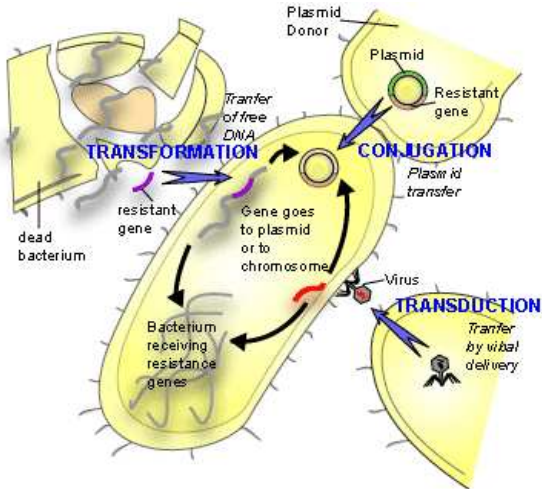


Figure 2: Mechanisms of antimicrobial resistance
Figure. 3: Forms of transfer of genetic material

Forms of resistance transfer

- Bacteria can spread their resistance factors vertically and horizontally. Vertically means that bacteria transfer their resistance by selection and multiplication of the chromosomal DNA bacteria that harbours point mutations (clonal transmission). Horizontal transmission is transmission between several different bacteria. Some examples of horizontal transmission mechanisms:
  - Conjugation (transfer of plasmids true bacterial pilli)
  - Transformation (acquiring external DNA from its surroundings)
  - Transduction (transfer by viral delivery)

The influence of the population on transfer of antimicrobial resistance

Stimulated by the increase of antimicrobial use worldwide, the occurrence of antimicrobial resistance in multiple bacterial populations escalated, especially in developing countries where antimicrobials were readily available without prescription (Levy and Marshall 2004). Restrictive usage of antimicrobials contributes to a decrease of antimicrobial resistance.

Fluoroquinolones and modern 3rd en 4th generation cephalosporins are classified by WHO to be of critical importance to human health. The result is that usage of the drugs in animals is restricted to clinical severe indications, preferably in individual animals. However, because of lack of control in the past decades these drug classes have been used preventively in food-producing animals, pets, consumption fish and ornamental fish. Reports on acquired resistance mechanisms have been published in increasing frequencies. Because of the importance to humans health, and misusage, our study focuses at these drug classes and resistance developed against them.

Fluoroquinolones

Fluoroquinolones include the older generation quinolones used in animals like oxolinic acid and flumequine and the second generation fluoroquinolones like enrofloxacin. The latter drug is closely related to ciprofloxacin, a drug commonly used in humans for treatment of infectious diseases.

According to the increase of quinolone usage, resistance among gram negative bacteria against quinolones increased as well. The classical know resistance mechanisms is a single point mutation in the quinolone resistance determinant regions (QRDR) of the gyrase or topoisomerase genes involved in cell multiplication. The mutation(s) result in a step wise decrease in susceptibility. Now a days it is also known that bacteria can exchange horizontaly transmissable quinolone resistance genes, which can facilitate the development of genetic mutations in the chromosomal DNA. (Strahilevitz, Jacoby et al. 2009). These genes include *qnr*, *qepA,* and *Aac(6')-1b-cr.*

In parts of South-East Asia and China, 60-70% of *E- coli* bacteria are resistant against fluoroquinolones. In the U.S. resistance is also rising against 10%, which could jeopardize the value of quinolones as a therapeutic antimicrobial drug (Karlowsky, Kelly et al. 2002), (Zervos, Hershberger et al. 2003).

Quinolones are excreted unaltered and are very persistent in the environment. Oxolinic acid has a half time value of 150-1000 days in the environment (Goni-Urriza, Capdepuy et al. 2000), (Halling-Sorensen, Nors Nielsen et al. 1998).

Developments in antibiotics

Any new developed antimicrobial drug should be very restrictively used, and as last resort. Taken the enormous costs of development, it's not profitable for pharmaceuticals to develop a drug that can only be used in last resort. The cooperation of science and government should result in the development of last resort antimicrobial drug, for the benefit of veterinary, and in particular human medicine. The registration of antimicrobial use must be endorsed, for reduction to be focused on whom it concerns the most and where most of the reduction is feasible. Reduction in use of antimicrobials in veterinary medicine can't be significant enough without focus on animal husbandry adjustments.

Fish bacteria

Aeromonas spp.

General

Aeromonas is a gram-negative, facultative anaerobic rod, belonging to the family of Aeromonadaceae. The bacteria are ubiquitous in fresh and brackish water.

Pathogenicity

Aeromonas species are in general pathogenic to cold blooded animals (fish, amphibians and reptiles), *Aeromonas* organisms are found as normal flora in nonfecal sewage, and are isolated from tap water, canals, streams and rivers (Feigin 2004). Despite the ubiquitous presence, and the fact that *Aeromonas* are mostly secondary pathogens, there is a big variability in virulence between strains, and some strains may become primary pathogenic. . Related to fish, factors often present in case of infection are high numbers of fish in a small tank, environment or poor water conditions, and mechanical injuries in the fish after netting or grading. For man, *Aeromonas* organisms may become a primary pathogen too, like with acute fulminating metastatic myositis (Feigin 2004).

Antimicrobial susceptibility

Aeromonas have shown to be resistant against penicillin's, and quinolones. Resistances against quinolones are due to mutations in the *gyrA* region of the QRDR. Mutations in *Aeromonas* strains have been found at codon 83 only so far (Goni-Urriza, Arpin et al. 2002).
Aeromonas isolates selected from fish farms show the same *gyrA* mutations over a period of several years, indicating a possible persistence of resistant strains on the fish farms (Giraud 2004).

Shewanella spp.

General

Shewanella spp. are gram-negative rods and belong to the family of Shewanellaceae. The *Shewanella* genus is a diverse group of marine gamma-proteobacteria, ranging from psychrophilic, to piezophilic, to psychrotolerant, to mesophillic ((Kato and Nogi 2001)).

Pathogenicity

Shewanella putrefaciens is somehow known in fish, and has been isolated so far from fish with fin rot, haemorrhages in de mouth, and popeye (Austin & Austin, 1999). *Shewanella denitrificans* are rare in man, from more or less severe clinical cases, sometimes with pneumonia and septicemia.

Citrobacter spp.

General

Citrobacter spp. are gram negative, motile and facultative anaerobic bacteria, belonging to the family of Enterobacteriaceae. *Citrobacters* are found not only in water, but also in soil, food and occasionally in the gastrointestinal tract of humans and animals (Arens and Verbist 1997).

Pathogenicity

In fish citrobacters are not known as primary pathogenic bacteria, they probably have a denitrifying function in the aquarium, and can be found in multibacterial skin lesions (own experience, CVI Lelystad). In man Citrobacters may cause urinary tract, respiratory, intrabdominal, wound, bloodstream and central nervous system infections (Samonis, Karageorgopoulos et al. 2009).

Antimicrobial susceptibility

In *C. freundii*, as in *E. coli*, the primary quinolone resistance mechanisms seems to be located in *gyrA* and *parC* (Navia, Ruiz et al. 1999). *C. freundii* strains have inducible ampC genes encoding resistance to ampicillin and cephalosporins (CMY). These CMY-type beta-lactamases have been transferred to other Enterobacteriaceae through plasmid conjugation and occur currently widespread in *E. coli*. In addition, isolates of *Citrobacter* may be resistant to multiple other antibiotics as a result of plasmid-encoded resistance genes.

Ornamental fish industry

Sector

The ornamental fish industry can be divided into three groups:

- Pond
- Freshwater tropical (aquarium)
- Marine water (Seawater, aquarium)

An estimate of 1-1,5 billion ornamental fish are traded annual internationally (Ploeg 2007; Whittington and Chong 2007). These includes about 4000 freshwater species and 1400 marine water species. (Whittington and Chong 2007). The trade of ornamental fish is the biggest of al companion animals in the world (Ministerie van LNV 2002). Ornamental fish farms produce 95% of the fish, the residual 5% is wild caught. (Althoff 2001; Raad voor Dierenaangelegenheden 2006; Ministerie van LNV 2008).

Tabel 1. Aantal gezelschapsdieren in Nederland

Diersoort /-groep	Aantal dieren (miljoen)	Index[1]	Jaar	Ref.
Katten	3,3	1.05	2005	25
Honden	1,8	1.04	2005	25
Konijnen	0,98	1.15	2003	16
Knaagdieren	0,80	0.85	2003	16
Zang- en siervogels	3,4	1.00	2003	16
Postduiven	1,15[2]	1.00	2005	6
Reptielen en amfibieën	0,25	1.00	2005	13[3]
Aquariumvissen	8,2	0.95	2003	16
Vijvervissen	10,8	0.94	2003	16
Totaal	**30,7**			

[1] t.o.v. 2 jaar ervoor.
[2] Gemiddeld aantal duiven: 1 miljoen ouderdieren in januari tot 2.3 miljoen in augustus met jongen.
[3] Schatting Reptielenzoo Serpo, Delft [13].

In the Netherlands an estimate of 19 million ornamental fish are kept, divided in 8,2 million aquarium fish and 10,8 million pond fish (DiBeVo 2010). The international trade in ornamental fish represents a total value of 2,2 billion US dollar. This is just 15% of the total amount representing the ornamental fish retail industry, containing products like aquaria, pumps and other peripherals, summing up to 15 billion US dollars a year (Ploeg 2007).

An estimate of 12-20 million ornamental fish are imported into the Netherlands each year, divided into 1500-1700 shipments (Ministerie van LNV 2002; Raad voor Dierenaangelegenheden 2006) from 41 countries (Althoff 2001). On the other hand, also 10 million fish are being exported every year (Postma 2005). Other estimates suggest that pond fish alone sells 10-30 million a year (Zwieten 1998). 1-2 Million are farmed in the Netherlands (Zwieten 1998), the rest is farmed mainly in Singapore, Brazil, US, Israel, Italy, Japan and Hong-Kong (Zwieten 1998; Ministerie van LNV 2002). More recent figures show that discus fish, Koi and goldfishes are increasingly imported from Malaysia and Singapore, these countries are the biggest exporting countries of the World on this moment (Ministerie van LNV 2008). Over 80% of Malaysian ornamental fish production is exported to Singapore (Ministerie van LNV 2008). More expansive Koi contributes for about 10% to international trade and are mainly exported from Japan (Zwieten 1998). In about 7% of the shipments problems occur, concerning unsound packaging or transport issues (Althoff 2001; Ministerie van LNV 2002).

Complete and consistent statistics concerning ornamental fish import into the Netherlands isn't available. (Ministerie van LNV 2002; Ploeg 2007). Data concerning species, amounts, health- and welfare status aren't well documented (Ministerie van LNV 2002). The statistics made available by the CBS are based on an estimation of the amount of fish, calculated from total weights of packaging, water and fish (Ministerie van LNV 2002).

Shipments of fish are often treated with some sort of drugs, whether it is to calm down the fish, improve water quality, prevention of infection or increase of oxygen intake (Ministerie van LNV 2002). It is known that the addition of disinfectants and/or antimicrobials is often applied (Cole 1999; Koene 2009).

There is a total of about 180 companies importing ornamental fish, of which 5-10 are big players (Postma 2005). 20 Wholesale companies represent 80% of the ornamental fish retail industry (Zwieten 1998). Because of transportation and stress, the fish are susceptible to infections. This explains why the wholesale companies are such an important piece of the puzzle, concerning the use of antimicrobial drugs. The use and discharge into the environment of animal drugs used in the ornamental fish industry isn't well documented as well as the extraction of the drug in sewages (Postma 2005).

Legal aspects of the ornamental fish industry

Since there is little legislation for husbandry, breeding and trading ornamental fish, a code of conduct is being developed to guarantee a durable way of breeding and trading. The development of this code was scheduled for 2008, yet to this moment no code has been published. The Ornamental Aquatic Trade Association (OATA) has made a code of conduct, which could be of guidance.

For importing of ornamental fish, legislation has been developed by the European Union through "2006/656/EC: animal health conditions and certification requirements for imports of ornamental fish". Control to implementation of this legislation is poor , taken the lack of good registration on import data concerning ornamental fish transport (Ministerie van LNV 2002).
The average age of a goldfish in the Netherlands has been estimated at 3-6 months, while this could be 20-30 years under good conditions (Zwieten 1998; Ministerie van LNV 2002).
As will be discussed under legislation, there are some veterinary drugs not subjected to the canalization regime, because they are covered by the so called 5 gram-regulation. This regulation implies the following:

> *"Veterinary antimicrobial drug and other resistance-inducing veterinary drug, exclusively suitable and destined for administering to aquarium- or terrarium animals, can be sold in packaging which contains at the most 5 grams of active substance"* (Ministerie van LNV 2010).

This regulation justifies the selling of antibiotics in pet stores, garden malls and similar stores. Internet is another easy gateway to get antibiotics for the treatment of ornamental fish

Antimicrobial usage and resistance in aquaculture

Use of antimicrobials in the ornamental fish industry

An average size retail company would use an estimated 50 kg of antibiotics a year (Postma 2005). There are no exact numbers to indicate the exact use of antimicrobials in the ornamental fish industry, suggestion is that the use of antimicrobials is widespread and more than once not through the consult of a veterinarian (Koene 2009). Reliable estimations cannot be made, because there is no good registration on the use of antimicrobials in the ornamental fish industry. Antimicrobials are put in transport water of ornamental fish during flight and transport to prevent die of and infection (Dixon 1990; Postma 2005). Restrictive use of antibiotics isn't promoted by the free market of several antimicrobials and other drugs for ornamental fish in pet stores and on the internet because of the 5 gram regulation.

These drugs contain:

- Dimetridazol
- Enrofloxacin
- Sarafloxacin
- Acriflavine

Other drugs used in the ornamental fish industry:

- Oxytetracycline
- Doxycycline
- Tetracycline
- Neomycin
- Amikacin
- Kanamycin
- Chloramphenicol
- Oxolinic acid
- Amoxycillin
- Trimethoprim-Sulfa
- Ceftiofur
- Florfenicol

Taken the lack of registered drugs for ornamental fish, every application is off-label. Since it isn't required to register or reveal the use of drugs in the ornamental fish industry, there is no good insight in water and drug use or the discharge of wastewater. The use and discharge into the environment of animal drugs used in the ornamental fish industry isn't well documented as well as the extraction of the drug in sewages (Postma 2005).

The antimicrobial concentrations in wastewater of an ornamental fish wholesale company showed significant differences compared to that of consumption fish farms (Postma 2005). Also several investigations report an increase in the prevalence of resistant bacteria isolated from water, sediments or wild animals near fish farms (Schmidt, Bruun et al. 2000; Giraud 2004).

Near to fish farms for consumption fish, antimicrobial concentrations couldn't be measured in the surface water. At the other hand, the surface water near ornamental fish farms, from which some excrete waste water direct into the surface water without purification, concentrations of dozens of µg/l were measured (table 2). This resulted in high concentrations of antibiotics downstream in the surface water (Postma 2005).

Fish have often been exposed to stress due to transport and handling, are being kept in large numbers in small tanks with sometimes several species in the same tank. Infections can flare up rather easily, frequently halted by using broad spectrum antibiotics (Cole 1999; Koene 2009).

Table 2. Human and veterinary drugs in the Netherlands in surface- and waste water in µg/ml.
RIZA rapport 2003-023

µg/L	Waste water				Surface water
	Hospital	Trout farm	Eelfarm	Ornamental fish commerce	Ornamental fish commerce
Erythromycine	0,3 - 1,1	-	-	0,7/0,8	-
Chlooramfenicol	6.5	-	-	0,37/1,8	12
Cefuroxime	-	-	-	-	-
Flumequine	-	-	-	3 /11	17
Ciprofloxacin	6,5 - 240	-	-	4.5	-
Enrofloxacin	-	-	-	12 /41	64
Clindamycine	6 - 32	-	-	4,9/12	4.9
Lincomycine	-	-	-	11/12	14
Oxytetracycline	-	-	-	16/120	57

Legislation

There are some veterinary drugs not subjected to the canalization regime, because they are covered by the so called 5 gram-regulation. This regulation implies the following:

> *"Veterinary antimicrobial drug and other resistance-inducing veterinary drug, exclusively suitable and destined for administering to aquarium- or terrarium animals, can be sold in packaging which contains at the most 5 grams of active substance"* (Ministerie van LNV 2010).

This regulation justifies the selling of antibiotics in pet stores, garden malls and similar stores. Internet is another easy gateway to get antibiotics for the treatment of ornamental fish. This is legal, if the five gram regulation is followed.
The number of kilograms used isn't comparable with the amounts used in farm animals, yet the induction of resistance is being endorsed by wrong or ignorant use of antibiotics.
Also there is loads of interaction between consumer and aquaria and exposure to possible pathogenic bacteria, which increases the potential risks. There is a proven correlation between keeping ornamental fish and the presence of multiple resistance *Salmonella* Java in young children (Musto, Kirk et al. 2006), which endorses these potential risks.

Figuur 1. **CASCADE naar EU-model.**

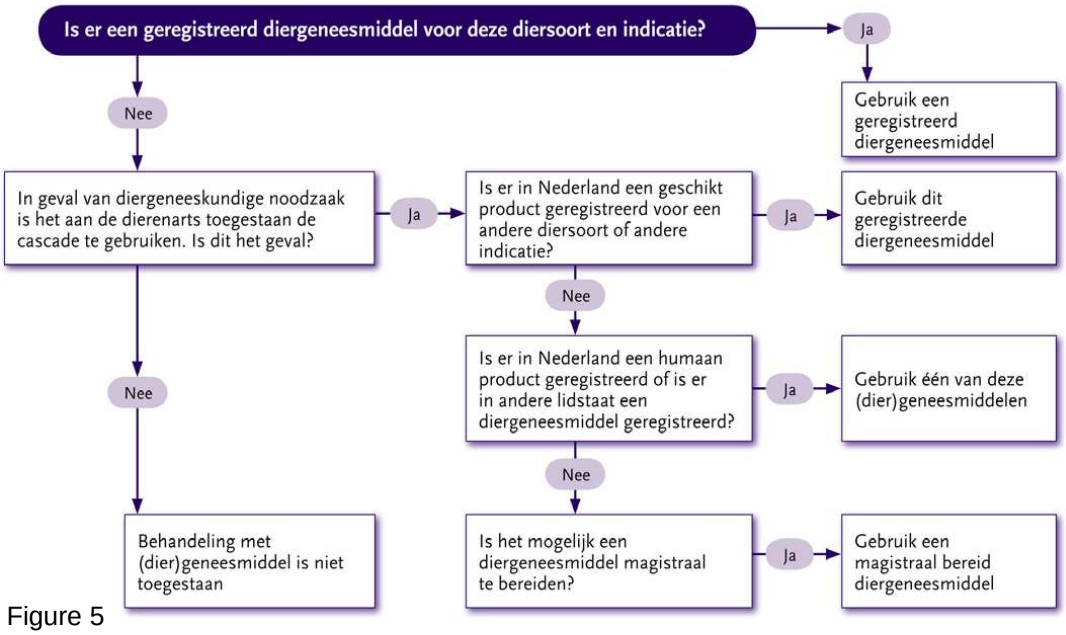


Figure 5
The cascade control presented by the KNMvD

The use of antibiotics, as practiced with knowledge and competence, can contribute to a decrease in the induction of antimicrobial resistance. The 5-gram regulation doesn't contribute to the goals of restrictive use lead by knowledge and competence, and can lead to wrong and unnecessary use of antibiotics. It has been demonstrated that the use of antibiotics for disease prevention or as antimicrobial growth promotion, can lead to an increase of antimicrobial resistance and resistance plasmids (Miranda, Kehrenberg et al. 2003; Nawaz, Sung et al. 2006; Das, Saha et al. 2009). Veterinarians are bound to the use a so called "Cascade-control" for using not licensed drugs in food animal treatments (fig. 5). Because there are no drugs registered for ornamental fish, there should be a veterinary necessity. Cases of a veterinary necessity should be about individual patients, yet ornamental fish are often treated as a group using tank medication. Water medication results in exposure of all fish in the tank, including healthy fish. Water medication for prevention of infection during transport or in quarantine, isn't included by the cascade regulation and should therefore not be applied by a veterinarian. In case there is a veterinary necessity, there should be looked for a drug similar, but registered to another species. In case not available you can apply a drug registered for humans or a veterinary drug registered in another European Union state. In last resort a veterinarian can choose to magisterial prepare the drugs.

Antimicrobial resistance in aquaculture

Antimicrobials in waste water are increasingly found and potentially have an important role in the rise and selection of antimicrobial resistance in the environment (Kummerer and Henninger 2003). Water borne organisms can act as a reservoir for resistance genes (Zhao and Aoki 1992).

There are significantly more resistant *Aeromonas* detected in samples of outlet water, compared to inlet water of some fresh water fish farms (Schmidt, Bruun et al. 2000) indicating some sort of selection on the way. A clear correspondence has also been demonstrated between the presence oxytetracycline-, trimethoprimsulfa- en oxolinic acid resistant *Aeromonas* spp.in the surroundings of fresh water fish farms (Schmidt, Bruun et al. 2000). Also after processing waste water by sewage processing plants elevated amounts of resistant bacteria can occur in rivers (Goni-Urriza, Capdepuy et al. 2000).

In 2009 Čížek et al. have done a similar investigation, where large number of resistant isolates where found. 72 Isolates of *Aeromonas* taken from ornamental carps where tested, whereof 50% was resistant to oxytetracycline, 25% was resistant to ciprofloxacin, 7% was resistant to chloramphenicol and florfenicol and 15% was resistant to trimethoprim.
They also compared the presence of antimicrobial resistance between ornamental carps and farmed carps, resulting in significantly more resistant bacteria isolated from ornamental carps. This is thought to be caused by the difference in legislation between ornamental- and farmed fish (Čížek, Dolejská et al. 2010). Warm water ornamental fish and their carriage water therefore contain more resistant bacteria compared to cold water ornamental fish and their carriage water (Verner-Jeffreys, Welch et al. 2009).

As (Giraud 2004) suggest, policies are needed to regulate the ever increasing use of veterinary fluoroquinolones in aquaculture.
The occurrence of resistance against quinolones in *A. salmonicida* is mainly linked to the acquisition of mutations in the *gyrA* gene (Giraud 2004).

The presence of a fresh water fish tank appears to be a risk factor in the infection with a multi-resistant form of *Salmonella* Java, especially for kids under 5 years of age (Musto, Kirk et al. 2006). Also, multi-resistant *Salmonella* Java has been found in weaned calves, showing great genetic similarity to *Salmonella* Java found in infections after contact with aquaria (Evans, Davies et al. 2005).

Research of (Verner-Jeffreys, Welch et al. 2009), has demonstrated that resistance has been globally distributed, yet *Aeromonas* spp. are good sensitive to 3rd an 4th generation cephalosporins. The same research demonstrated that ornamental fish and their carriage water can act as a reservoir for multi-resistant bacteria and their genes (Verner-Jeffreys, Welch et al. 2009). Antimicrobial resistance can be transported between different Aeromonads and other bacteria, through class 1 integrons (Chang, Shih et al. 2007).

Resistance against several antibiotics probably finds an important part of its origin in the use of them in the past. Against oxytetracycline, nitrofuranes, potentiated sulfonamides and oxolinic acid loads of resistance has been seen (Schmidt, Bruun et al. 2000; Musto, Kirk et al. 2006; Verner-Jeffreys, Welch et al. 2009; Čížek, Dolejská et al. 2010). These antimicrobial drugs have been used a lot for years during transport and for the prevention of losses (Dixon 1990).

Pathogenicity in humans

Several water borne Aeromonads (such as *A. veronii, A. hydrophila* and *A. caviae*) can cause gastro-enteritis with severe diarrhea in children and immune compromised people. These infections can be so serious that antimicrobial treatment is necessary (Vila, Ruiz et al. 2003; Rathinasamy Subashkumar 2006). If infection with multi-resistant Aeromonads occurs, antimicrobial therapy won't give the desired result or will take longer due to the search for an effective antibiotic.

Vibrio vulnificus as a cause of wound infection, sepsis or gastro-enteritis isn't very common in the Netherlands and Europe. Taken the high and fast mortality rate for sepsis (50%), a quick and effective treatment is of great importance. Infections often take place in warm climate countries, yet the increase of warm water fish farms with recirculated water may contribute to more infections in the Netherlands (Dijkstra 2009). Since there is a high mortality rate, a slow and non-effective treatment against multi-resistant bacteria can be lethal or life threatening.

There is evidence for the ornamental fish tanks to be reservoir for some multi-resistant types of *Salmonella Paratyphi* B variants, resulting in infection in especially younger children (Levings, Lightfoot et al. 2006). Since there is also a connecting between keeping ornamental fish and the presence of multi-resistant Salmonella Paratyphi B variant Java in young children (Musto, Kirk et al. 2006) this forms a potential human health risk.

The spread of resistance and the numerous possibilities of transmission of resistance determinants between bacteria causes resistance to be a great risk for human- and veterinary healthcare (Levy and Marshall 2004).

In humans Citrobacters may cause urinary tract, respiratory, intrabdominal, wound , bloodstream and central nervous system infection (Samonis 2009).

Shewanella denitrificans are rare in man, from more or less severe clinical cases, sometimes with pneumonia and septicaemia.

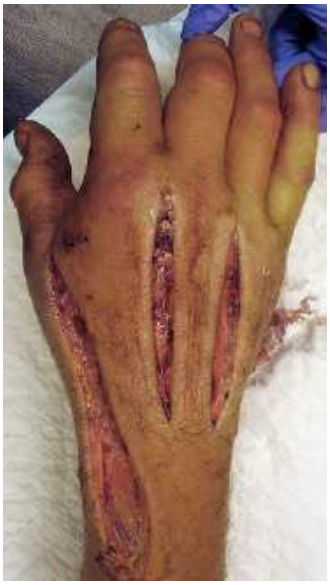


Figure 6
Bear claw incisions made for treatment of Fasciitis necroticans due to *Vibrio vulnificus* from (Dijkstra 2009)

Aim of the pilot/Hypothesis

Preface

The ornamental fish sector is a globally growing market, in which very few data are available. This makes it difficult to make accurate estimations of the use of antimicrobials and other drugs that can be used to treat or prevent infections. Loads of fish are being imported from Singapore, Japan and Malaysia. To prevent damage and die-off during transport, antibiotics are used in for instance transport water. During section and bacterial examination at the CVI, multi-resistant aeromonads, *Vibrio* and *Shewanella* are frequently isolated from ornamental fish.
How big the problem of resistance in ornamental fish really is and what the consequences could be for the ornamental fish industry, the veterinary- and human healthcare isn't known due to the lack of data. To make a start estimating the size of the problem, this pilot study has been set up to estimate the prevalence of antimicrobial resistance in bacteria from ornamental fish. Besides susceptibility testing there has also been looked after the presence of resistance genes, focused on quinolone and cephalosporin resistant bacteria.

Aim of the pilot

The aim of the pilot study is to investigate the presence and identity of resistance genes in a collection of multi-resistant *Aeromonas*, *Vibrio* and *Shewanella* with extra attention to resistance against quinolones and cephalosporins.

Hypothesis

Since the intensive use of antibiotics worldwide in the ornamental fish trade it is expected that transmissible resistance genes may be present in bacteria isolated from ornamental fish. Thereby, antimicrobial resistance would be a big problem to fish and man, as many people have direct contact with the water their ornamental fish lives in.

Materials and methods

Collection and identification of isolates

Twenty seven bacteria isolated from ornamental fish (Koi, Discus fish, Goldfish) at the CVI where selected out of 237 strains isolated in the past five years and kept at –80 ℃ suspended in peptone glycerol medium. These bacteria were typed biochemically, which was confirmed by 16S rDNA typing. The selection was based on susceptibility/resistance patterns determined by disk diffusion method used for diagnostic purposes at CVI.

The isolates were first identified based on their growth and biochemical characteristics using classical microbiological methods.

- Growth at 37 ℃.
- Motility, using a motility test medium
- Oxidase, tested with a culture grown from Tryptose agar
- Katalase, tested with a culture grown from Tryptose agar. All negative reactions were checked with a microscope.
- Dextrose, oxidative/fermentative, and gas formation
- Nitrate, de-nitrification

Subsequently all isolates were identified by a sequence based method, where a sequence analysis is performed on a PCR product of the highly conserved and species specific 16S rDNA to measure its base pare structure. The results of the sequence analysing can be BLASTed using the NCBI database.

16S rDNA typing

16S rDNA typing has been performed on all bacteria according to protocol 00-14-0872 version 1
At first, samples were prepared for PCR amplification. From a fresh bacterial culture on agar, the full contents of a disposable loop (1 µl) of bacterial growth was suspended in 1 mL SQ water and vortexed. After a 5 minute centrifuge at 13000 RPM the bacterial pellet was re-suspended in 100 µL SQ water. After a 10 minutes heating at 100C in a water bath, the culture was diluted 1/100 in SQ water and used as sample for the PCR amplification.

PCR amplification of DNA sample

15 µL of the diluted (1/100) DNA sample was added to 15 µL PCR Master mix. The acquired samples (30 µL) were put into the PCR GeneAmp PCR system 9700 The amplification was performed in following steps: 10' at 95 ℃, followe d by 30 cycles of 30"at 95 ℃, 30" at 60 ℃ and 45" at 72 ℃, after these cycles 10' at 72 ℃ is fo llowed by cooling down to 4 ℃. After this PCR amplification, the acquired product must be purified before sequence PCR can be performed.

Purification of PCR product

For purification of the PCR products the Qiaquick PCR purification kit (Qiagen) was used according producers protocol.

PCR sequence-run, forward en reverse

13 µL forward sequence master mix and 13 µL reverse sequence master mix are being supplemented with 7µL purified PCR product.
The acquired samples (20 µL) are being put into the PCR device, where the "16S rDNA sequence PCR" program performs a sequence determination in 25 runs at following temperatures: 25 cycles of 10" 96 ℃, 5" 50 ℃ and 4" 60 ℃, followed by a cooling down to 4 ℃. After this sequence PCR, the product is ready to be prepared for sequence analyzing.

Sequence analysing by the Applied Biosystems 3130 Genetic Analyser

After the sephadex has been welling for a minimum of 3 hours with 300 µL SQ water in a M96 membrane plate, it has been centrifuged into a M96 base point plate at 2000 RPM for 5 minutes. The sequence PCR product has been pipetted unto the sephadex, followed by centrifugation for another 5 minutes at 2000 RPM into a clean base point plate. The centrifuged product has been pipetted from the M96 base point plate unto an M 96 sequence plate, which can then be prepared for analysing by the sequence analyzer.

The sequence analyzer is being operated using the users protocol, provided with the machine. For typing following program has been used: **Fast seq.36 pop7_v1.1**
Files produced by the analyzer can then be analyzed by MicroSeg or Sequence analyser using either the program database (Microseq) or the NCBI database (Sequence analyzer, BLAST) to compare the obtained sequences with known sequences of different bacteria species.

Antimicrobial susceptibility testing: disk diffusion test

All 27 bacterial isolates were tested for antimicrobial susceptibility using the disk diffusion test according to CLSI guidelines. From a fresh pure bacterial culture on blood agar plates, a suspension of 0.5 McFarland was prepared in a physiological salt solution (0.85% NaCl). Pre-dried Mueller Hinton agar plates were inoculated with the 0.5 McFarland suspension using cotton swabs. After inoculation paper disks were applied. The Mueller-Hinton plates where incubated at 22 degrees Celsius. After incubation inhibition zone diameter was measured at 24 and 48 hours.
The antibiotic paper disks contained the following antibiotics:

- Oxolinic acid
- Cefotaxim
- Oxytetracycline
- Ampicillin

The inhibition zones where read by two persons, independently from each other. The averages were taken from these two measurements.
The following criteria where held to determine sensible (S) or resistant (R):
Oxolinic acid: < 17 mm = R, > 17 mm = S
Cefotaxim: < 24 mm = R, > 24 mm = S
Oxytetracycline: < 24 mm = R, > 24 mm = S
Ampicillin: < 20 mm = R, > 20 mm = S

MIC determination

For a selection of 12 isolates that were classified resistant to oxolinic acid based in disk diffusion, the resistance levels were quantified by the determination of the minimum inhibitory concentrations (MIC). MIC of the following antimicrobials where tested:
Ampicilin, chloramphenicol, ciprofloxacin, colistin/polymixin B, florfenicol, cefotaxime, gentamicin, kanamycin, nalidixic acid, sulfonamide, streptomycin, ceftazidime, tetracycline and trimethoprim.
A 0.5 Mc Farland suspension was prepared of a one day old culture, if poorly grown after one day we used a two day old culture. After a dilution of 1/200 in (Cation Adjusted Mueller Hinton Broth (CAMHB) the inoculum was pipetted into a 96 wells microtiter plate (format: EUMVS2, Trek Diagnostic Systems) and incubated for 24 hours for MIC determination. The cultures which have proved to grow at 37 ℃, where tested at 35 ℃, the cultures which did not grow at 37 °Celsius where tested at 22 °Celsius for 24 hour. If growth was insufficient for determination, plates where additionally incubated another 24 hours.
As quality control strains *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* ATTC 33658 Were included at both incubation temperatures (22 °C and 35 ℃).

ESBL determination

These isolates were subsequently tested for susceptibility to a panel of cefalosporins with the same method using ESBF1 plates, to study the presence of ESBL. ESBL determination was performed using following antimicrobials: ampicilin, cefriaxone, cephalotin, ciprofloxacin, cefotaxim/clavulanic acid, cefazolin, cefepime, cefotaxime, cefoxitin, gentamicine, imipenem, meropenem, piperacillin/tazobactam, cefpodoxime, ceftazidime/clavulanic acid and ceftazidime.

Determination of QRDR mutations

Selected bacterial isolates, based on their quinolone resistance pattern where tested for the presence of mutations in the QRDR regions of *gyrA* and *parC* with earlier described sets of primers specially designed for *Aeromonas* spp. We started with these primers to determine the optimal MgCl2 concentration (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM). The *gyrA* PCR on the *Aeromonas* samples was performed with 3,0 mM MgCl2 and the *Citrobacter* and *E. coli* samples with 2,0 mM MgCl2. The *parC* PCR gave best results using 2,0 mM MgCl2.
Before purification of the PCR product, gel electrophoresis was performed on all samples to make sure the PCR reaction was successful and the amplicon had the right size. *gyrA* :441 base pairs (bp) and *parC* :204 bp).

Performing PCR for *gyrA* of *Aeromonas* we used a primer mix of 5 µL PCR buffer, 1 µL forward or reverse *gyrA* primer, 5 µL dNTP's (2.0 mM), 6 µL $MgCl_2$ (3mM), 0.5 µL AmpliTaq, supplemented with 26.5 µL SQ water resulting in 45 µL mix, which is complemented with 5 µL sample lysate. The 50 µL product was used to perform the PCR in following steps: 5' at 95 ℃, followed by 35 cycles of 1' 94C, 1' 55C and 1' 72C. After these cycles 10' of 72C is followed by cooling down to 4C.
Performing PCR for *parC Aeromonas* the same mix was used, except we altered the concentration of $MgCl_2$ to 4 µL and an extra 2 µL of SQ water to keep a total of 45 µL mix. The same amount of lysate was used and also PCR steps were the same. We tried different annealing temperatures from 51 to 59 degrees ℃, in steps of 2 degrees to determine best product for sequence usage.
Al samples that gave a satisfactory PCR product, where submitted to a sequence analyzing. Before analyzing, purification was performed and gel electrophoresis was performed to make sure product s where present.

Purification of PCR product

Before performing a sequence PCR, all products must be purified first, to prevent debris being multiplied. For purification of the PCR product the Qiagen Qiaquick PCR purification kit was used, according to manufacturer's protocol.
After purification, the amount of PCR product is measured either with measuring by a Nano-Drop ND-1000 (NanoDrop Technologies Inc.), by gel electrophoresis or both.

PCR sequence-run, forward en reverse

After purification a Sequence PCR was performed using a PCR master mix, containing 1µL PCR buffer,5 µL *SQ,* 1µL PCR product, 1µL primer (forward or reverse), 2 µL Big Deye Terminator v1.1
The acquired samples (10 µL) are being put into the PCR device, in following steps: 1' of 96℃ is followed by 25 cycles of 10" at 96℃, 5" at 50℃ an d 4' at 60℃, followed by cooling down to 4℃.

Sequence analysing by the Applied Biosystems 3130 Genetic Analyser

After the sequence PCR, the acquired product can be prepared for analysing with the sequence analyser.
After the sephadex has been welling for a minimum of 3 hours with 300 µL SQ water in a M96 membrane plate, it has been centrifuged at 2000 RPM for 5 minutes and collected in a microtiter plate, a 96 well V-shaped micro plate.
First, 10 µl sterile water was added to the PCR product. Subsequently, the total volume (20 µl) was pipetted unto the sephadex, followed by centrifugation for another 5 minutes at 2000 RPM and collected in a sterile microtiter plate a clean base point plate. The centrifuged product was pipetted from the 96 well V-shaped micro plate into a M 96 sequence plate, which can then be prepared for analysing by the sequence analyser.
The results of the sequence analyser can be BLASTed after editing by a sequence analysing program.

To detect point mutations in the QRDR-region, specific primers for *gyrA* and *parC* area designed for *Aeromonas* were used (Goni-Urriza, Arpin et al. 2002). Sequences of the used primers are showed in table *.

gyrA	Asal *gyrA* F	TCCTATCTTGATTACGCCATG	58–78
	Asal *gyrA* R	CATGCCATACCTACCGCGAT	520–539
parC	Ec *parC* F	GAAACCTGTTCAGCGCCGCAT	139–159
	Ec *parC* R	TTCGGTGTAACGCATTGCCGC	371–391

Table 3 Primers used for PCR of the *gyrA* and *parC* region of the QRDR.

For PCR 25mM dilutions where used, for sequence PCR 10mM of the same primers (forward and reverse) where used.

Micro array

All isolates for which MICs were determined the presence of resistance genes commonly present in Enterobacteriaceae was studied using a commercial miniaturized microarray (Identibac AMR-ve). Using this array a wide variety of resistance gene families can detected including resistance against aminoglycosides, quinolones, beta-lactams, trimethoprim, sulfonamides and macrolides.

A 10 µL loop of freshly overnight grown bacteria was suspended in 400 µL lysis buffer and vortexed for about 5 seconds. Subsequently the suspension was incubated at 60℃ for 2 hours, and vortexed every 30 minutes. After incubation at 60℃, incubation at 95℃ occurs for 15 minutes. After this last heating, centrifugation at 13000 rpm for 5 minutes was performed. 6.45µL Supernatant (400-2000 ng/µL) was mixed with dNTP mix (1 µL), Therminator 10x amplification buffer (1 µL), Therminator DNA polymerase (0.1 µL), Biotin-16-dUTP (0.35 µL) and primer mix (1 µL)to create the mix used to perform the PCR. The following cycles are performed on this 10 µL reaction volume: 5 minutes at 96°, 40 cycles of 20 second at 62°, 40 seconds at 72°and 60 seconds at 96°, after which the product was cooled down to 4°.
Micro-array tubes (tubes) were being used and hybridization performed according to protocol of the manufacturer

ESBL confirmation

In the isolates with a positive signal for the CMY probes, the presence and characteristics of the gene was tested by specific PCR and subsequent sequence analysis of the amplicon. The primers used are listed in table 4.

primername:	**nucleotide sequence:**	**Size:**	
CMY-2 F	5'-ATG-ATG-AAA-AAA-TCG-TTA-TGC-TGC-3'	1117 BP	MedVetNet Workpackage-09
CMY-2 R	5'-GCT-TTT-CAA-GAA-TGC-GCC-AGG-3'		

Table 4
Primers used for PCR amplification of CMY

Screening of plasmid mediated quinolone resistance (PMQR)

In all isolates with a quinolone resistance phenotype, the presence of plasmid mediated quinolone resistance genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *and* *aac (6') 1b-* cr was studied using specific primers as listed in table 5.
In case of a positive PCR reaction, sequence analyzing was performed.

primername	nucleotide sequence	Size:	
qnrA-fw	5' GGA-TGC-CAG-TTT-CGA-GGA 3'	492 bp	(Cavaco, Frimodt-Moller et al. 2008)
qnrA-rev	5' TGC-CAG-GCA-CAG-ATC-TTG 3'		
qnrB-fw	5' GGM-ATH-GAA-ATT-CGC-CAC-TG 3'	263 bp	(Goni-Urriza, Arpin et al. 2002)
qnrB-rev	5' TTT-GCY-GYY-CGC-CAG-TCG-AA 3'		
qnrB-F	5'- GAT-CGT-GAA-AGC-CAG-AAA-GG-3'	469 bp	(Gay, Robicsek et al. 2006)
qnrB-R	5'- ACG-ATG-CCT-GGT-AGT-TGT-CC-3'		
qnrC-fw	5' GGG-TTG-TAC-ATT-TAT-TGA-ATC 3'	447 bp	(Wang, Guo et al. 2009)
qnrC-rev	5' TCC-ACT-TTA-CGA-GGT-TCT 3'		
qnrD-fw	5' CGA-GAT-CAA-TTT-ACG-GGG-AAT-A 3'	582 bp	(Cavaco, Hasman et al. 2009)
qnrD-rev	5' AAC-AAG-CTG-AAG-CGC-CTG 3'		
qnrS-fw	5' TCG-ACG-TGC-TAA-CTT-GCG 3'	467 bp	(Cavaco, Frimodt-Moller et al. 2008)
qnrS-rev	5' GAT-CTA-AAC-CGT-CGA-GTT-CGG 3'		
qepA-F	5'-TGG TCT ACG CCA TGG ACC TCA-3'	1137 bp	(Perichon, Courvalin et al. 2007)
qepA-R	5'-TGA ATT CGG ACA CCG TCT CCG-3'		
aac(6')-Ib-F	5'-TTG-CGA-TGC-TCT-ATG-AGT-GGC-TA-3'	482 bp	(Park, Robicsek et al. 2006)
aac(6')-Ib-R	5'-CTC-GAA-TGC-CTG-GCG-TGT-TT-3'		

Table 5
Primers used for PCR amplification of the PMQR genes

For all strains for which a specific amplicon was obtained this amplicon was purified and the electrophoresis repeated, before sequence analysis was performed.

Results

To get familiar with classical typing, we performed several tests on all isolates, as described in materials and methods. Finally we determined al samples using 16S rDNA identification, resulting in the names listed in table 6.
After determination all samples were submitted to a disk susceptibility test, using the four antibiotics described in table 6. All measurements were taken after 48 hours, because some samples showed poor growth after 24 hours. All pink numbers in the table are resistant against the specific antibiotic, according to CLSI guidelines. After these disk susceptibility tests, a selection was made to perform more specific tests on. Selected samples are made yellow. Sample 20A was a sample selected from the inhibition zone to cefotaxime.

Samples project	DSU-number	Species	Origin/organ	Bacterial typing	Oxolinic acid	Oxytetracyclin	Ampicilin	Cefotaxim
					OA	OT	AMP	CTX
					48h	48h	48h	48h
1	9015306	Koi	Skin	Aeromonas veronii	0	0	0	34
2	9015306	Koi	Skin	Shewanella putrefaciens	0	0	24,5	30
3	09015972-1	Koi	Organs	Aeromonas hydrophila	0	8	0	32,5
4	09015972-2	Koi	Organs	Aeromonas veronii	0	0	0	37,5
5	10009061-1	Koi	Organs/Skin	Shewanella putrefaciens	0	0	22,5	34
6	10012578-1	Koi	Skin	Aeromonas veronii	0	8	0	35,5
7	6033894	Discusfish	Organs	Edwardsiella tarda	40	40	37,5	40
8	7027830	Koi	Organs/Skin	Aeromonas salmonicida	40	40	40	40
9	9007549-6	Koi	Skin	Aeromonas salmonicida	19,5	40	21	39
10	9007549-14	Koi	Skin	Acidovorax temperans	40	40	40	40
11	5000052	Koi	Skin	Aeromonas salmonicida	19	11	15	28
12	4023556	Koi	Skin	Aeromonas salmonicida	19	19,5	14	30
13	5001185-2	Koi	Skin	Aeromonas salmonicida	40	40	40	40
13.2 MH	5001185-2	Koi	Skin	Aeromonas salmonicida	14	40	25	40
13.2 IST	5001185-2	Koi	Skin	Aeromonas salmonicida	0	28	19	17
14	5006527	Koi	Skin	Aeromonas salmonicida	16	40	18	27
15	6015106	Goldfish	Skin	Acidovorax delafieldie/facilis	0	0	0	30
16	583816	Discusfish	Spleen	Aeromonas punctata	0	7	23,5	35
17	584806	Koi	Skin/intestine	Aeromonas hydrophila	0	8	0	38
18	586806	Carp	Skin	Pseudomonas fluorescens	0	21,5	0	0
19	587255	Koi	Skin	Aeromonas salmonicida	19,5	17,5	19	25
20	533696	Koi	Filter	Citrobacter freundii	0	0	0	29
20A	533696	Koi	Filter	Citrobacter freundii	0	0	0	0
21	534211	Koi	nb	Aeromonas hydrophila	0	8	0	37,5
22	539016	Trout	Furunculose	Aeromonas salmonicida	38	37	36	40
23	x			Aeromonas veronii	0	0	0	29,5
24	553326	Koi	Skin	Aeromonas spp.	0	0	0	30,5
25	10015213-1	Poecelia		Shewanella putrefaciens	0	0	22	36
A. salmonicida ATTC 33658				According to reference	40	46	35	39
Yellow = selected for MIC								

Table 6
Typing and disk susceptibility test results

After the selection MICs were determined for ampicilin, chloramphenicol, ciprofloxacin, colistin/polymixin B, florfenicol, cefotaxime, gentamicin, kanamycin, nalidixic acid, sulfonamide, streptomycin, ceftazidime, tetracycline and trimethoprim. The complete results of these MIC determination are shown in table 12 in the Appendix. Most frequent occurred resistance to nalidixic acid with 100% (12), followed by tetracycline 75% (9), sulfonamide 75% (9), Ampicillin 50% (6), cefotaxime 42% (5), trimethoprim 42% (5,)kanamycin 33% (4), chloramphenicol 25% (3), ciprofloxacin 25% (3), florfenicol 25% (3), gentamicin 17% (2) and ceftazidime 8% (1).

The results of the ESBL determination, performed using a list of beta-lactam antibiotics (appendix, table 13 suggest the presence of classical ESBL and/AmpC-type beta-lactamases.

Micro array

All selected samples, used for the MIC and ESBL determination were tested with a micro array for the presence of additional resistance genes. In table 7 all genes with array values above 0,30 are shown. Values between 0.3 and 0.4 indicate an ambiguous result. Values > 0.4 are positive.

Sample	Name	Micro array Genes with values of 0,30 and higher						
1	***Aeromonas veronii***	tetE						
2	***Shewanella putrefaciens***	tetA	tetD	intI1	aadA2	floR	dfr12	sul1
3	***Aeromonas hydrophila***	tetA						
11	***Aeromonas salmonicida***	tetD	ereB					
13	***Aeromonas salmonicida***	strB						
14	***Aeromonas salmonicida***							
15	***Acidovorax delafieldie/facilis***	tetA	tetC	IntI	aadA*	aac6Ib	sul1	
16	***Aeromonas punctata***	tetA	IntI	aadA2	dfr12	sul1	ereB	
18	***Pseudomonas fluorescens***							
18A	***Pseudomonas fluorescens***							
20	***Citrobacter freundii***	sul2	tetD	cmy	qnrB	blaCMY		
20A	***Citrobacter freundii***	sul2	tetD	cmy	qnrB	blaCMY		
25	***Shewanella putrefaciens***	tetA	catB3	floR	dfr12	cmy	sul1	ereB
E-coli 2	**ATCC 25922**	ereB						
E-coli	**ATCC 25922**							
Aer. salmonicida 2	**ATCC 33658**	ctxM1*	Act1*	qnrS	strA*			
Aer. salmonicida	**ATCC 33658**	sul2	ctxM1	act1	qnrS	strA	blaMOX-CMY9*	

* = value between 0,30 and 0,45

Table 7
Values micro array above 0.30

Table 7 shows that genes encoding for tetracycline resistance (tet) were most commonly present. Moreover genes encoding for resistance to streptomycin (str, aadA2), kanamycin (aac61B), florfenicol (floR), sulfonamides (sul), trimethoprim (dfr), and chloramphenicol (cat) were detected. To our surprise we detected a qnrB gene in strain 20/20A, next to a CMY-gene.
The negative controls (*A. salmonicida* ATCC 33658) were tested twice, because of their positive tests in the first array. To make sure the *qnrS* gene was an aspecific reaction, al samples were tested for specific PMQR genes later on. Complete results of the micro-array can be found in table 14 in the appendix.

Quinolone resistance

The same selection was used for detection of mutations in the Quinolone Resistance Determining Region (QRDR). We amplified the gyrA and *parC* genes using PCR, the samples mentioned in table 8 were those on which an amplicon was produced . Sequence analysis of all samples in table 8 was successful for the *gyrA*, yet the *Shewanella* couldn't yet be compared to a control *gyrA* gene. The *parC* sequence analysis failed numerous times, except for both *Citrobacter* samples. Apparently, the primers we used were not adequate for this spcies.
The *Aeromonas* samples showed several point mutations. The mutation Ser-83-Arg is most commonly described in literature, Ser-83-Ile and Leu-92-Met are more rare mutations (Goni-Urriza, Arpin et al. 2002).
Based on the mutations, the MICs and disk susceptibility test results combined, there could be a correlation between the number of point mutations and the quinolone susceptibility, looking at an increased MIC for ciprofloxacin in case of 3 mutations. Yet one sample with 3 mutations is not enough to draw conclusions.

We also tested for plasmid mediated quinolone resistance by targeted PCR aimed at the following genes: *qnrA, qnrB, qnrC, qnrD, qnrS, qepA,* CMY and *aac(6') 1b-cr.*
Al samples generating a product of primer specific size where purified and it's sequence was analysed after a second gel electrophoresis.

Tested samples:

1	Koi	Aeromonas veronii
2	Koi	Shewanella putrefaciens
3	Koi	Aeromonas hydrophila
11	Koi	Aeromonas salmonicida
13	Koi	Aeromonas salmonicida
14	Koi	Aeromonas salmonicida
15	Goldfish	Acidovorax delafieldie/facilis
16	Discusfish	Aeromonas punctata
18	Carp	Pseudomonas fluorescens
20	Koi	Citrobacter freundii
20A	Koi	Citrobacter freundii

Table 8
Samples tested for the presence of PMQR genes

In *Citrobacter* we found a common (Gay, Robicsek et al. 2006) point mutation Thr-83-Ile and the presence of a *qnrB* resistance gene, encoding for quinolone resistance. Based upon the least silent mutations, *qnrB-9* is a possible type, determined by BLAST in the NCBI database. Other possibilities are shown in table 15 in the appendix.

Samples	Bacterial typing	Mutations						MIC		Disk susceptibility test
		Gyr A				Par C	PMQR	CIP	Nal	Oxolinic Acid (48h)
1	Aeromonas veronii	3	Ser-83-Ile	Leu-92-Met	Arg-137-Stop	?		0,50	> 64	*0*
2	Shewanella putrefaciens	?				?		> 8,00	64	*0*
11	Aeromonas salmonicida	1	Ser-83-Arg			?		0,12	> 64	19
13	Aeromonas salmonicida	1	Ser-83-Arg			?		0,12	64	*0*
14	Aeromonas salmonicida	1	Ser-83-Arg			?		0,12	64	*16*
20	Citrobacter freundii	1	Thr-83-Ile			0	*qnrB*	0,50	> 64	*0*
20A	Citrobacter freundii	1	Thr-83-Ile			0	*qnrB*	0,50	> 64	*0*
E-coli	ATCC 25922	-				-		<= 0,008	<= 4,00	
A. salmonicida	ATCC 33658	-				-		<= 0,008	<= 4	40

Table 9
Samples, mutations, PMQR results and quinolone resistance combined

In sample 15 (Acidovorax) we found a *aac(6') 1b* variant. Sequencing revealed that this gene was not the ciprofloxacin resistant (cr) PMQR variant and conferred only resistance to kanamycin.

ESBL identification

Sequence analysis of the *Citrobacter* isolate with a positive signal in the microarray test for the CMY probe showed that the gene was CMY 2 with 3 silent mutations.

Discussion and recommendations

It must be considered that this study was a pilot. We looked into a broad diversity of bacteria from fish, and made a selection based on the antibiotics used in routine diagnostic tests at our laboratory. After that, we selected further, based on cefotaxime and quinolone resistance. Only a few strains from all strains isolated in the CVI-lab, gathered in the last decade were in this selection.
We found a CMY-2 variant, with 3 silent mutations in *Citrobacter freundii*. This CMY-2 is known as the most prevalent *AmpC* gene among Enterobacteriaceae (Su et al 2006) and commonly detected on conjugative plasmids. However, in *Citrobacter* it is present on the chromosomal DNA and therefore only vertically transferable by clonal distribution.
In epidemiologic surveys around the world *qnrA*, *qnrB* and *qnrS* were found in several Enterobacteriaceae, including *Citrobacter freundii* (Ferreira et al 2010). Most quinolone resistant bacteria are resistant because of point-mutations in the QRDR of the topoisomerase genes, as we saw in *Aeromonas* spp. In *Citrobacter* we also found a *qnrB* gene, combined with a common mutation in the *gyrA* region.
The presence of these resistance patterns in bacteria from ornamental fish needs careful interpretation. Although *Citrobacter freundii* has been isolated in hospital infections (Ferreira et al 2010), this does not necessarily mean that the same species isolated from ornamental fish has a zoonotic potential because humans and fish bacteria could even within one species belong to different variants that are not directly epidemiologically related to each other. However, exchange of resistance genes between *Citrobacter freundii* strains is possible.
Qnr-genes as the one we detected in a *Citrobacter* are located on a plasmids and can therefore easily be transferred to other Enterobacteriaceae, like was described for *qnrA* by Nordmann et al in 2005.
Aquarium water, with or without antimicrobials in it, are drained into surface water or sewage plants, which are ideal sites for exchange of antimicrobial resistance genes, because there is a constant influx of enteric bacteria of large numbers are present and in close contact with each other. Moreover, fluoroquinolones are detected in wastewater in high concentration and and are persistent enough to create new resistances, when transferred into the environment.

In the Netherlands the implementation of legislation for the application of antimicrobials in animals is not fully adequate. Currently there is an ongoing debate about a reduction of the use of antimicrobials prescribed by veterinarians, used for farm or to a lesser extend also companion animals. For ornamental fish a so called five gram regulation exists, which allows sales of antibiotics for use in fish in small amounts by all kinds of stores without prescription by a veterinarian. This may result in improper and random usage of antimicrobials like tetracycline and enrofloxacine. This regulation results in the use of antimicrobials, randomly applied by amateurs without specific knowledge. After application this waste water will be drained into the sewage systems. Via contact with sewage water humans may be at risk. The magnitude of the risk is likely to be small, as sewage is not in contact much with man, but it may affect the evolution of resistant organisms in the surface water and the environment.

Given the enormous imports and transfers of ornamental fish from all over the world, and the fact, that preventively many antibiotics are used to avoid disease during these transports, there is a true the risk for fish hobbyists to get into contact with resistant bacteria via their imported ornamental fish and fish tank water. Via this route humans will be in contact with resistance bacteria and/or genes, which may add to public health risks. If the bacteria and/or genes would be an infectious multiresistant bacteria, like the *Citrobacter*, these infections would be more difficult to cure. That this possible route of transmission is not science fiction but reality is demonstrated by the detection of genetically related multiresistant *S*. Java in children obtained from (ornamental) fish and aquarium or tank water and weaned claves (Musto, Kirk et al. 2006). Genetically related *S.* Java was detected in calves in the United Kingdom as well, suggesting an epidemiological relation with the isolates from fish (Evans, Davies et al. 2005).

Apart from the risk of acquiring resistance genes, man may be severely infected by these organisms as well. At an eel farm for edible fish production, an eel farmer obtained a direct zoonotic infection from his diseases eels, which suffered a *Vibrio vulnificus* outbreak (Dijkstra 2009), with a bacterial strain which was multiresistant to antibiotics. From ornamental aquaria, zoonotic infections to man are known like fish tuberculosis, caused by *Mycobacterium marinum*, which causes fish tank granuloma in man (Austin B , Austin DA (1987) Bacterial Fish Pathogens. Ellis Horwood Ltd, Chichester). This fish disease is very common in ornamental fish all over the world, and can mostly not be cured. However, fish hobbyists do not like to lose their expensive fish, and use long-term treatments with antibiotics. As this infection in fish is chronic it would need months of antibiotic treatment, if effective at all, and this would easily induce resistant genes. As antibiotics are free available via the five gram regulation, it would be recommended, that all antibiotics be solely on prescription via a veterinarian, to avoid selection of resistance.

In this pilot we used several methods to identify and characterize the resistance phenotypes observed. Therefore we were able to confirm or exclude some initial results. The microarray showed a *qnrS* positive signal for the *Aeromonas salmonicida* control sample, yet by PCR using specific *qnrS* primers did not confirm the presence of a *qnrS* in the control *Aeromonas*. This shows that indicative data always need to be confirmed before final conclusions are drawn on presence or absence of genes.

The g*yrA* mutations at positions 83 and 92 of the QRDR of the topoisomerase genes are published in other articles, yet the mutations at position 137 are new and should be confirmed by a second or third sequence analysis, to verify that we found a new mutation instead of a misreading. We can confirm that the quinolone resistance we found, as found in literature, is based on mutations in the *gyrA* region.

Citrobacters like all Enterobacteriaceae often contain several plasmids coding for antimicrobial resistance. Since we have tested only one *Citrobacter* and detected a plasmid mediated *qnrB*-gene, interpretation of this outcome cannot be anything more than that the presence such a gene in this single sample. However, this indicates that these genes are commonly present and can be easily detected, if looked for. To determine whether more citrobacters, originating from ornamental fish and their surroundings, contain corresponding plasmids, further investigation of these bacteria is needed.
Because of the finding of bacteria resistant to cefotaxime using antimicrobial disk susceptibility testing, it is advisable to start screening for cephalosporin resistance on a regular base. This way a emerging of more cephalosporin resistance can be detected and extra information of resistance patterns obtained.

Although we tested only a small selection of bacteria out of out of 237 bacteria isolated from ornamental fish, we cannot say, that the results are not important: the laboratory of CVI only gets a small amount of all bacterial disease problems in ornamentals in our country – often people just buy new fish and discharge their diseased fish, or buy antibiotics via the five grams regulation. Therefore, the results suggest as a warning, there are resistance genes present in ornamental fish, and urge us to look for further genes.

Conclusion

In this pilot we conducted an extended screening, looking at several different more and less pathogenic bacteria isolated from lesions of ornamental fish, We conclude that multiresistance is present in many of these strains against several antimicrobial classes commonly used in animal and human medicine. Also resistance to antimicrobials considered of critical importance to human health can easily be detected in these bacteria. Especially quinolone resistance is present in *Aeromonas* spp. due to mutations in the *gyrA* region of the QRDR. Not only primary fish pathogens contained resistance factors: *Citrobacter freundii* contained mutations in the *gyrA* of the QRDR, *qnrB* and CMY in one sample. No real ESBL's were found in bacteria from ornamental fish, but we found transmissible quinolone resistance genes. This implies a possible threat for the spread of organisms or plasmids harbouring resistance genes to other gram negatives in animals or humans. Given the frequent and close contact of humans with ornamental fish and waste water from this industry, and the intensive use of antibiotics in ornamental fish, an exchange of resistance to humans must be considered to be a potential public health risk.
Taken into account the small number of samples we tested, this study indicates a broad presence of antimicrobial resistance in ornamental fish. Therefore, it is concluded that active surveillance of resistance genes of concern for public health like ESBL's and plasmid mediated quinolone resistance genes should be conducted routinely. Also the free purchase by the public, of antimicrobials for ornamental fish, legalized by the five gram regulation should be restricted.

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Appendix

Complete results

		Properties					
Sample	Name after typing	Growth on 37℃	Motility	Oxidase	Katalase	Dextrose	Nitrate
1	Aeromonas veronii	1	0	1	1	11	1
2	Shewanella putrefaciens	1	1	1	1	0	1
3	Aeromonas hydrophila	1	1	1	1	11	1
4	Aeromonas veronii	1	1/0 *	1	1	11	0
5	Shewanella putrefaciens	0	1	1	1	0	1
6	Aeromonas veronii	1	1	1	1	11	1
7	Edwardsiella tarda	1	1	0	1	11	1
8	Aeromonas salmonicida	0	0	1	0	10	0
9	Aeromonas salmonicida	0	0	1	0	10	1
10	Acidovorax temperans	0	0	1	1	0	1
11	Aeromonas salmonicida	0	0	1	0	10	1
12	Aeromonas salmonicida	0	0	1	0	10	1
13	Aeromonas salmonicida	0	0	1	0	10	1
14	Aeromonas salmonicida	0	0	1	0	10	1
15	Acidovorax delafieldie/facilis	1	0	1	1	0	1
16	Aeromonas punctata	0	1	1	1	10	1
17	Aeromonas hydrophila	0	1	1	1	11	1
18	Pseudomonas fluorescens	1	1	1	1	11	1
19	Aeromonas salmonicida	0	0	1	0	10	1
20	Citrobacter freundii	0	1	0	1	11	1
21	Aeromonas hydrophila	0	1	1	1	11	
22	Aeromonas salmonicida	0	0	1	1	11	
23	Aeromonas veronii	1	1	1	1	11	
24	Aeromonas spp.	1	1	1	1	11	

0 = negative
1 = positive
*: no motility in test medium, yet motility seen under microscope

Table 10
Results of classical typing. 11 = fermentative and oxidative.

Antimicrobial disk susceptibility test;

agar diffusion testing according to CLSI guidelines (Table 11)

										Accoording to CLSI criteria				
Samples project	DSU-number	Species	Origin/organ	Bacterial typing	Oxolinic acid	Oxytetracyclin	Ampicilin	Cefotoxim		Oxolinic acid	Oxytetracyclin	Ampicilin	Cefotoxim	
					OA	OT	AMP	CTX		OA	OT	AMP	CTX	Multiresistant
					48h	48h	48h	48h		48h	48h	48h	48h	Na 48 uur
1	9015306	Koi	Skin	Aeromonas veronii	0	0	0	34		R	R	R	S	3
2	9015306	Koi	Skin	Shewanella putrefaciens	0	0	24.5	30		R	R	S	S	2
3	09015972-1	Koi	Organs	Aeromonas hydrophila	0	8	0	32.5		R	R	R	S	3
4	09015972-2	Koi	Organs	Aeromonas veronii	0	0	0	37.5		R	R	R	S	3
5	10009061-1	Koi	Organs/Skin	Shewanella putrefaciens	0	0	22.5	34		R	R	S	S	2
6	10012578-1	Koi	Skin	Aeromonas veronii	0	8	0	35.5		R	R	R	S	3
7	6033894	Discusfish	Organs	Edwardsiella tarda	40	40	37.5	40		S	S	S	S	0
8	7027830	Koi	Organs/Skin	Aeromonas salmonicida	40	40	40	40		S	S	S	S	0
9	9007549-6	Koi	Skin	Aeromonas salmonicida	19.5	40	21	39		S	S	S	S	0
10	9007549-14	Koi	Skin	Acidovorax temperans	40	40	40	40		S	S	S	S	0
11	5000052	Koi	Skin	Aeromonas salmonicida	19	11	15	28		S	R	R	S	2
12	4023556	Koi	Skin	Aeromonas salmonicida	19	19.5	14	30		S	R	R	S	2
13	5001185-2	Koi	Skin	Aeromonas salmonicida	40	40	40	40		S	S	S	S	0
14	5006527	Koi	Skin	Aeromonas salmonicida	16	40	18	27		R	S	R	S	2
15	6015106	Goldfish	Skin	Acidovorax delafieldie/facilis	0	0	0	30		R	R	R	S	3
16	583816	Discusfish	Spleen	Aeromonas punctata	0	7	23.5	35		R	R	S	S	2
17	584806	Koi	Skin/intestine	Aeromonas hydrophila	0	8	0	38		R	R	R	S	3
18	586806	Carp	Skin	Pseudomonas fluorescens	0	21.5	0	0		R	R	R	R	4
19	587255	Koi	Skin	Aeromonas salmonicida	19.5	17.5	19	25		S	R	R	S	2
20	533696	Koi	Filter	Citrobacter freundii	0	0	0	29		R	R	R	S	3
20A	533696	Koi	Filter	Citrobacter freundii	0	0	0	0		R	R	R	R	4
21	534211	Koi	nb	Aeromonas hydrophila	0	8	0	37.5		R	R	R	S	3
22	539016	Trout	Furunculose	Aeromonas salmonicida	38	37	36	40		S	S	S	S	0
23	x			Aeromonas veronii	0	0	0	29.5		R	R	R	S	3
24	553326	Koi	Skin	Aeromonas spp. (hydrophila?)	0	0	0	30.5		R	R	R	S	3
25	10015213-1	Poecelia		Shewanella putrefaciens	0	0	22	36		R	R	S	S	2

MIC determination (Table 12)

Samples project	Bacterial typing	Grown at 22/35 °C	Measured at 24/48 h	Plate	Antibiotic											
					AMP	Ref AMP	CHL	Ref CHL	CIP	Ref CIP	COL	Ref COL	FFN	Ref FFN	FOT	Ref FOT
1	Aeromonas veronii	35	24	EUMVS2	> 32	R	<= 2	S	0.50	S	<= 2		<= 2	S	<= 0.06	S
2	Shewanella putrefaciens	35	24	EUMVS2	1	S	16	I	> 8.00	R	<= 2		16	R	0.12	S
3	Aeromonas hydrophila	35	24	EUMVS2	> 32	R	<= 2	S	0.50	S	> 4		<= 2	S	<= 0.06	S
11	Aeromonas salmonicida	22	48	EUMVS2	8	S	16	I	0.12	S	> 4		<= 2	S	2	I
13	Aeromonas salmonicida	22	**24**	EUMVS2	16	I	32	R	0.12	S	> 4		<= 2	S	4	R
13.1	Aeromonas salmonicida	22	**48**	EUMVS2	32	R	32	R	0.25	S	> 4		<= 2	S	4	R
14	Aeromonas salmonicida	22	48	EUMVS2	16	I	16	I	0.12	S	> 4		<= 2	S	4	R
15	Acidovorax delafieldie/facilis	35	48	EUMVS2	> 32	R	<= 2	S	> 8	R	<= 2		<= 2	S	4	R
16	Aeromonas punctata	22	24	EUMVS2	2	S	<= **2**	S	0.25	S	<= 2		<= 2	S	<= 0.06	S
18	Pseudomonas fluorescens	35	48	EUMVS2	> 32	R	32	R	0.06	S	<= 2		32	R	> 4	R
20	Citrobacter freundii	22	24	EUMVS2	8	S	8	S	0.50	S	<= 2		8	I	0.12	S
20A	Citrobacter freundii	35	24	EUMVS2	> 32	R	8	S	0.50	S	<= 2		16	R	> 4.00	R
25	Shewanella putrefaciens	35	24	EUMVS2	2	S	64	R	> 8.00	R	4		<= 2	S	<= 0.06	S
CLSI	Guidelines E-coli				2 - 8		2 - 8		0.004 - 0.015		0.25 - 2		2 - 8		0.03 - 0.12	
Control	E-coli	22	24	EUMVS2	4.00		8.00		<= 0.008		<= 2.00		8.00		0.12	
Control	E-coli	35	24	EUMVS2	4.00		8.00		<= 0.008		<= 2.00		8.00		0.12	
Control	A. salmonicida ATTC 33658	22	24	EUMVS2	<= 0.5		<= 2		<= 0.008		<= 2		<= 2		<= 0.06	
Number of resistant samples out of 12					10		6		3		0		4		5	
Percentage resistant					91%		55%		25%		0%		36%		42%	

R = Resistant
I = Intermediate
S = Susceptible

																		Multiresistant
GEN	Ref GEN	KAN	Ref KAN	NAL	Ref NAL	SMX	Ref SMX	STR	Ref STR	TAZ	Ref TAZ	TET	Ref TET	TMP	Ref TMP	NEG	POS	Out of 12
1,00	S	<= 4	S	> 64	R	<= 8	S	64	-	<= 0,25	S	16	R	1,00	S	OK	OK	3
0,50	S	> 128	R	64	R	512	R	32	-	<= 0,25	S	> 64	R	> 32,00	R	OK	OK	7
1,00	S	> 128	R	> 64	R	16	S	16	-	0,50	S	16	R	8,00	S	OK	OK	4
0,5	S	<= 4	S	> 64	R	1024	R	> 128	-	0,5	S	32	R	1	S	OK	OK	3
<= 0,25	S	<= 4	S	64	R	1024	R	> 128	-	8	I	<= 1	S	2	S	OK	OK	4
0,5	S	<= 4	S	64	R	1024	R	> 128	-	1	S	<= 1	S	2	S	OK	OK	5
<= 0,25	S	<= 4	S	64	R	1024	R	> 128	-	0,5	S	<= 1	S	1	S	OK	OK	3
16	R	64	R	> 64	R	1024	R	128	-	4	S	> 64	R	16	R	OK	OK	9
1,00	S	<= 4	S	64	R	1024	R	64	-	<= 0,25	S	16	R	> 32,00	R	OK	OK	4
<= 0,25	S	<= 4	S	32	R	32	S	<= 2	-	1	S	<= 1	S	> 32	R	OK	OK	6
0,50	S	<= 4	S	> 64	R	> 1024	R	16	-	<= 0,25	S	> 64	R	<= 0,50	S	OK	OK	3
0,50	S	<= 4	S	> 64	R	1024	R	16	-	> 16,00	R	> 64	R	<= 0,50	S	OK	OK	7
> 32,00	R	> 128	R	> 64	R	512	R	8	-	<= 0,25	S	> 64	R	> 32,00	R	OK	OK	8
0.25 - 1		1 - 4		1 - 4		8 - 32		geen criterium		0.06 - 0.5		0.5 - 2		0.5 - 2				
<= 0,25		<= 4,00		<= 4,00		<= 8,00		4,00		0,50		2,00		2,00		OK	OK	
0,50		<= 4,00		<= 4,00		<= 8,00		4,00		<= 0,25		<= 1,00		1,00		OK	OK	
1		<= 4		<= 4		128		8		<= 0,25		<= 1		<= 0,5		OK	OK	
	3		5		12		16		0		1		9		7			
	27%		45%		100%		145%		0%		8%		75%		64%			

		S	I	R
AMP	Ampicilin	<= 8	16	>= 32
CHL	Chlooramphenicol	<= 8	16	>= 32
CIP	Ciprofloxacin	<= 1	2	>= 4
COL	Colistin/Polymixin B	?	?	?
FFN	Florphenicol	<= 2	8	>= 16
FOT	Cefotaxim	<= 1	2	>= 4
GEN	Gentamycin	<= 4	8	>= 16
KAN	Kanamycin	<= 16	32	>= 64
NAL	Nalidixic acid	<= 16		>= 32
SMX	Sulfonamid	<= 256		>= 512
STR	Streptomycin	-	-	-
TAZ	Ceftazidim	<= 4	8	>= 16
TET	Tetracyclin	<= 4	8	>= 16
TMP	Trimetoprim	<= 8		>= 16

ESBL confirmation test (Table 13)

Samples project	Bacterial typing	Grown at 22/35 °C	Measured at 24/48 h	Plate	Antibiotic													
					AMP	Ref AMP	AXO	Ref AXO	CEP	Ref CEP	CIP	Ref CIP	F/C	Ref F/C	FAZ	Ref FAZ	FEP	Ref FEP
11	Aeromonas salmonicida	22	24	ESB1F	> 16		8		> 16		<= 1		<= 0.12/4		> 16		4	
13	Aeromonas salmonicida	22	24	ESB1F	16	I	16	I	16	R	<= 1		<= 0.12/4	R	> 16	R	4	S
14	Aeromonas salmonicida	22	48	ESB1F	16	I	4	S	> 16	R	<= 1		<= 0.12/4	R	> 16	R	<= 1	S
15	Acidovorax delafieldie/facilis	22	48	ESB1F	> 16	I	2	S	> 16	R	> 2		<= .12/4	R	> 16	R	<= 1	S
18	Pseudomonas fluorescens	22	24	ESB1F	> 16	I	64	R	> 16	R	<= 1		64 /4	R	> 16	R	8	I
20A	Citrobacter freundii	22	24	ESB1F	> 16	I	32	R	> 16.00	R	<= 1		16>4	R	> 16.00	R	<= 1.00	S
Control	E-coli 25922	22	24	ESB1F														
Control	Pseudomonas	22	24	ESB1F	> 16		> 128		> 16		<= 1		> 64/4		> 16		4	
Control	A. salmonicida 33658	22	24	ESB1F	<= 8		<= 1		<= 8		<= 1		<= 0.12/4		<= 8		<= 1	
Number of resistant samples					0		2		5		0		5		5		0	
Percentage resistant					0%		18%		45%		0%		45%		45%		0%	

R = Resistant
I = Intermediate
S = Susceptible

																	Multiresistant
FOT	Ref FOT	FOX	Ref FOX	GEN	Ref GEN	IMI	Ref IMI	MERO	Ref MERO	P/T	Ref P/T	POD	Ref POD	T/C	Ref T/C	TAZ	Ref TAZ	Out of 16
4		8		<= 4		<= 0,5		<= 1		> 64/4		32		<= 0.12/4		4		
16	S	8	S	<= 4	S	<= 0,5		<= 1	S	> 64 /4	R	8	S	<= 0.12/4	R	8	S	5
4	S	8	S	<= 4	S	<= 0,5	-	<= 1	S	<= 4/4	R	16	R	<= 0.12/4	R	8	S	6
0,5	S	<= 4	S	> 16	S	4,0	-	<= 1	S	<= 4/4	R	0,5	S	<= 0.12/4	R	1	S	5
64	R	> 64	R	<= 4	S	4,0	-	2	S	8/4	R	> 32	R	> 8>4	R	4	S	9
32	I	> 64	R	<= 4	S	<= 0,5	-	<= 1,00	S	16/4	R	> 32,00	R	32>4	R	32,00	R	9
> 64		> 64		<= 4		1		<= 1		16/4		> 32		16/4		8		
<= 0,25		<= 4		<= 4		<= 0,5		<= 1		<= 4>4		<= 0,25		<= 0.12>4		<= 0,25		
	1		2		0		0		0		5		3		5		1	
	9%		18%		0%		0%		0%		45%		27%		45%		9%	

AMP	Ampiciline	Penicilin
AXO	Cefriaxone	Cephalosporin III
CEP	Cephalotin	Cephalosporin I
CIP	Ciprofloxacin	Fluoroquinolone
F/C	Cefotaxime/clavulanic acid	
FAZ	Cefazolin	Cephalosporin I
FEP	Cefepime	Cephalosporin IV
FOT	Cefotaxime	Cephalosporin III
FOX	Cefoxitin	Cephamicins
GEN	Gentamycine	Aminoglycosine
IMI	Imipenem	Carbapenem
MERO	Meropenem	Carbapenem
P/T	Piperacillin/ Tazobactam	
POD	Cefpodoxime	Cephalosporin (oral)
T/C	Ceftazidime/clavulanic acid	
TAZ	Ceftazidime	Cephalosporin III

Complete result micro array (Table 14)

Signal Values

Stam	Naam	prob_sul2_10		prob_sul2_11		prob_sul3_11		prob_tetA_11		prob_tetB_11		prob_tetC_11		prob_tetD_1		prob_tetE_11		prob_tetG_11		prob_tetG_12		prob_intI1_1		prob_intI2_11		prob_aadA1_1	
1	Aeromonas veronii	0	-	0	-	0	-	0	-	0	-	0	-	0	-	750	R	0	-	0	-	0	-	0	-	0	-
2	Shewanella putrefaciens	0	-	0	-	0	-	650	R	0	-	0	-	650	R	0	-	0	-	0	-	660	R	0	-	0	-
3	Aeromonas hydrophila	0	-	0	-	0	-	770	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
11	Aeromonas salmonicida	0	-	110	S	0	-	0	-	0	-	0	-	320	R	0	-	0	-	0	-	0	-	0	-	0	-
13	Aeromonas salmonicida	0	-	180	I	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
14	Aeromonas salmonicida	0	-	120	S	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
15	Acidovorax delafieldie/facilis	0	-	0	-	0	-	480	R	0	-	450	R	0	-	0	-	0	-	0	-	630	R	0	-	0	-
16	Aeromonas punctata	0	-	0	-	0	-	820	R	0	-	0	-	0	-	0	-	0	-	0	-	670	R	0	-	0	-
18	Pseudomonas fluorescens	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
18A	Pseudomonas fluorescens	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
20	Citrobacter freundii	0	-	610	R	0	-	0	-	0	-	0	-	810	R	0	-	0	-	0	-	0	-	0	-	0	-
20A	Citrobacter freundii	0	-	670	R	0	-	0	-	0	-	0	-	780	R	0	-	0	-	0	-	0	-	0	-	0	-
25	Shewanella putrefaciens	0	-	0	-	0	-	725	R	225	I	125	S	0	-	0	-	0	-	0	-	125	S	0	-	0	-
Salmonella	ATCC stam	0	-	650	R	0	-	710	R	0	-	0	-	690	R	0	-	0	-	0	-	640	R	0	-	0	-
E-coli 2	ATCC stam	0		0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
E-coli	ATCC stam	0	-	0	-	0	-	125	S	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Aer. Salmonicida 2	ATCC stam	0		70	S	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Aer. Salmonicida	ATCC stam	0	-	300	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-

Stam	Naam	prob_aadA2_1		prob_aadA4_1		prob_ctxM1_11		prob_ctxM1_12		prob_cmlA1_11		prob_catA1_11		prob_catIII_1		prob_catB3_11		prob_floR_11		prob_dfrA1_21		prob_dfrA1_22		prob_dfrA7_11		prob_dfrA7_12		prob_dfr12_11
1	Aeromonas veronii	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
2	Shewanella putrefaciens	600	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	700	R	0	-	0	-	0	-	0	-	660
3	Aeromonas hydrophila	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
11	Aeromonas salmonicida	0	-	0	-	0	-	0	-	0	-	0	-	0	-	75	S	0	-	0	-	0	-	0	-	0	-	0
13	Aeromonas salmonicida	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
14	Aeromonas salmonicida	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
15	Acidovorax delafieldie/facilis	300	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
16	Aeromonas punctata	560	R	0	-	0	-	0	-	0	-	0	-	0	-	70	S	0	-	0	-	0	-	0	-	0	-	820
18	Pseudomonas fluorescens	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
18A	Pseudomonas fluorescens	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
20	Citrobacter freundii	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
20A	Citrobacter freundii	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
25	Shewanella putrefaciens	75	S	0	-	0	-	0	-	0	-	0	-	0	-	780	R	550	R	0	-	0	-	0	-	0	-	850
Salmonella	ATCC stam	0	-	0	-	775	R	0	-	0	-	0	-	0	-	0	-	760	R	0	-	0	-	0	-	0	-	0
E-coli 2	ATCC stam	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
E-coli	ATCC stam	0	-	0	-	0	-	260	I	0	-	0	-	0	-	75	S	180	I	0	-	0	-	0	-	0	-	0
Aer. Salmonicida 2	ATCC stam	0	-	0	-	325	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
Aer. Salmonicida	ATCC stam	0	-	0	-	630	R	0	-	0	-	0	-	50	S	0	-	0	-	0	-	0	-	0	-	0	-	0

Signal value of
0-150 = S
150-300 = I
>300 = R
<50 = counted as 0

CENTRAL VETERINARY INSTITUTE
WAGENINGEN UR
Matthijs van Waart (0249254)

prob_dfrA7_12		prob_dfr12_11		prob_dfr13_11		prob_dfrA14_21		prob_dfrA15_1		prob_dfrA17_11		prob_dfrA19_1		prob_aac3la_1		prob_aac3IVa_1		prob_aac6lb_1		prob_ant2la_1		prob_act1_11		prob_dha1_1	
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	50	S	0	-
0	-	660	R	675	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	25	S	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	575	R	0	-	0	-	0	-
0	-	820	R	50	S	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-		-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	850	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	800	R	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	R	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	R	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	R	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	410	R	0	-
0	-	0	-	R	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	750	R	0	-

prob_acc2_11		prob_mox_1pm		prob_mox_1mm		prob_cmy_11		prob_fox_11		prob_tem1_1		prob_oxa1_21		prob_oxa2_11		prob_oxa7_11		prob_ctxM9_11		prob_shv1_11		prob_oxa9_11		prob_len1_11	
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	25	S	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	110	S	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	850	R	230	I	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	880	R	275	I	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	725	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	150	I
0	-	0	-	0	-	0	-	0	-	800	R	0	-	0	-	0	-	0	-	610	R	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	125	S	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	125	S	0	-	0	-	0	-	0	-	0	-	0	-

prob_acc1_11		prob_ctxM2_11		prob_per2_1		prob_dfrV_21		prob_sul1_11		prob_pse1_1pm		prob_pse1_1mm		prob_catB8_12		prob_ctxM26_11		prob_ctxM8_11		prob_qnrB_11		prob_qnrB_12		prob_qnrS_11	
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	700	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	750	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	725	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-		-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	800	R	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	850	R	0	-
0	-	0	-	0	-	120	S	710	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	160	I	775	R	0	-	0	-	0	-	0	-	0	-	0	-	775	R	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	80	S	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	430	R
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	710	R

prob_qnr_12		prob_gapA_611		prob_ihfA_611		prob_strA_611		prob_strB_611		hp_blaCMY_611		hp_blaCMY_612		hp_blaMOX-CMY9_611		hp_blaMOX-CMY9_612		hp_blaMOX-CMY9_613		hp_dnaE_611		hp_dnaE_612		hp_dnaE_613	
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0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	280	I	0	-	0	-	0	-
0	-	0	-	0	-	50	S	225	I	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	125	S	530	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	70	S	260	I	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
0	-	400	R	780	R	0	-	0	-	850	R	0	-	0	-	0	-	0	-	0	-	0	-	100	S
0	-	430	R	830	R	0	-	0	-	850	R	0	-	0	-	0	-	0	-	0	-	0	-	150	I
0	-	0	-	0	-	0	-	0	-	0	-	100	S	0	-	0	-	0	-	0	-	0	-	0	-
0	-	125	S	675	R	350	R	725	R	0	-	0	-	0	-	0	-	0	-	720	R	0	-	670	R
0	-	110	S	520	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	610	R	375	R
0	-	110	S	710	R	0	-	0	-	0	-	110	S	0	-	0	-	0	-	0	-	760	R	750	R
0	-	60	S	0	-	400	R	0	-	0	-	0	-	180	I	60	S	0	-	0	-	0	-	0	-
0	-	0	-	0	-	675	R	0	-	0	-	0	-	400	R	150	I	0	-	0	-	0	-	0	-

hp_ereA_611		hp_ereA_612		hp_ereB_611		hp_ereB_612		hp_ermB_611		hp_ermB_612		hp_hemL_611		hp_hemL_612		hp_hemL_613		hp_hemL_614		hp_vatE_611		hp_vatE_612		0,1M NaPP Standard pH 9		Biotin-		Aantal boven 300	Aantal tussen 150 en 300
0	-	0	-	60	S	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	750	OK	1	0
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0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	760	OK	0	1
0	-	0	-	150	I	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	760	OK	6	1
50	S	0	-	600	R	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	820	OK	6	0
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0	-	0	-	725	R	0	-	0	-	0	-	0	-	300	R	0	-	0	-	0	-	0	-	0	-	850	OK	8	2
575	R	740	R	300	R	0	-	0	-	0	-	460	R	0	-	130	S	0	-	0	-	0	-	0	-	800	OK	20	1
0	-	0	-	620	R	0	-	0	-	0	-	0	-	360	R	0	-	0	-	0	-	0	-	0	-	770	OK	7	0
0	-	0	-	0	-	0	-	0	-	0	-	50	S	600	R	0	-	0	-	0	-	0	-	0	-	850	OK	6	2
0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	780	OK	6	1
160	I	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	850	OK	8	2

Quinolone resistance

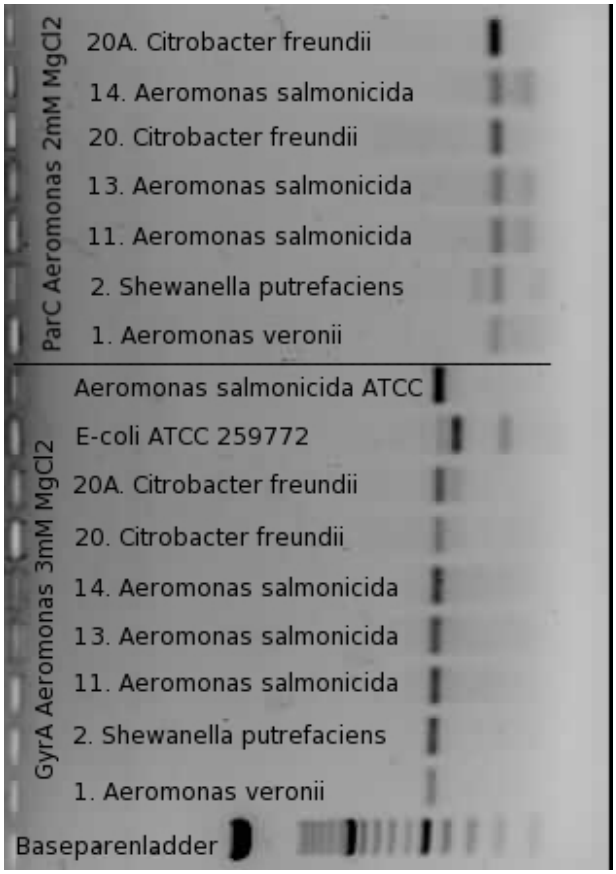

Figure 7
Results gel electrophoresis for both *gyrA* and *parC*. A 100 DNA molecular weight marker is used.

Mutations QRDR *gyrA*

Sample 1 Aeromonas veronii
Control L47978
CCGCACGGCGACA**GT**GCCGTGTATGACACCATTGTCCGC**TT**GGCGCAGGATTTC
1F
CCGCACGGCGAT**AT**CGCTGTGTATGACACCATTGTCCGG**A**TGGCGCAGGATTTC
1R
CCGCACGGCGAT**AT**CGCTGTGTATGACACCATTGTCCGG**A**TGGCGCAGGATTTC

Mutation Ser-83-Ile en Leu-92-Met

Control L47978 CGAGCTGCTGGC**C**GATCTGGACAAAGA
1F CGAGCTGCTGGC**T**GATCTGGACAAAGA
1R CGAGCTGCTGGC**T**GATCTGGACAAAGA

Mutation op Arg-137-Stop

Sample 11 Aeromonas salmonicida

```
Control L47978   GCACGGCGACAGTGCCGTGTATGACAC
11F              GCACGGCGACAGAGCCGTGTATGACAC
11R              GCACGGCGACAGAGCCGTGTATGACAC
```

Mutation Ser-83-Arg

Sample 13 Aeromonas salmonicida

```
Control      CCGCACGGCGACAGTGCCGTGTATGAC
13-R         CCGCACGGCGACAGAGCCGTGTATGAC
13-F         CCGCACGGCGACAGAGCCGTGTATGAC
```

Mutation Ser-83-Arg

Sample 14 Aeromonas salmonicida

```
Control      CCGCACGGCGACAGTGCCGTGTATGAC
14-R         CCGCACGGCGACAGAGCCGTGTATGAC
14-F         CCGCACGGCGACAGAGCCGTGTATGAC
```

Mutation Ser-83-Arg

Aeromonas salmonicida ATCC control sample

```
Control L47978      83                                        92
CCGCACGGCGACAGTGCCGTGTATGACACCATTGTCCGCTTGGCGCAGGATTTC

Asal F
CCGCACGGCGACAGTGCCGTGTATGACACCATTGTCCGCTTGGCGCAGGATTTC

Asal R
CCGCACGGCGACAGTGCCGTGTATGACACCATTGTCCGCTTGGCGCAGGATTTC

                                  137
Control L47978   CGAGCTGCTGGCCGATCTGGACAAAGA
Asal F           CGAGCTGCTGGCCGATCTGGACAAAGA
Asal R           CGAGCTGCTGGCCGATCTGGACAAAGA
```

Sample 20 Citrobacter freundii

```
Control      ACC----------GAC
20-R         ATCGCCGTTTACGAC
20-F         ATCGCCGTTTACGAC
```

Mutation Thr-83-Ile

Sample 20A Citrobacter freundii

```
Control      ACC----------GAC
20-R         ATCGCCGTTTACGAC
20-F         ATCGCCGTTTACGAC
```

Mutation Thr-83-Ile

In samples 20 and 20A (*Citrobacter*) we found a variant of *qnrB*. Depending on the number of silent point mutations, following *qnrB* types are possible:

Type *qnrB*:	Number of silent mutations:
qnrB 6	11
qnrB 9	1
qnrB 14	9
qnrB 18	7
qnrB 23	8

Table 15
Possible *qnrB* types, depending on the number of silent mutations

aac (6'), CMY and *qnrB* produced a product of the expected size, made visible using gel electroforesis, as seen below.

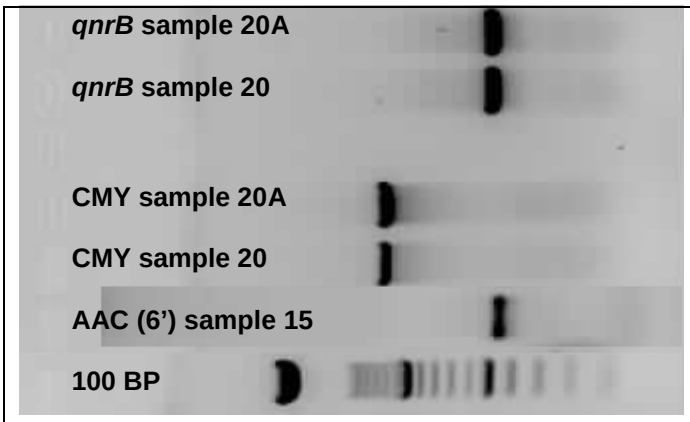

Figure 8
Results of the gel electrophoresis for the purified products used for sequence analyzing.