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Antimicrobial resistant *E. coli* and enterococci in pangasius fillets and prawns in Danish retail imported from Asia.

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Abstract

Antimicrobial resistance (AMR) genes, resistant bacteria and antimicrobial residues may be transferred to humans through consumption of fish and prawns raised in aquaculture. This study investigated AMR in *E. coli* and enterococci introduced to Denmark via prawns and pangasius products imported from Asia. In total, 300 samples of frozen pangasius fillets and prawns were collected from retail shops around Denmark. Samples were collected every two months between September 2017 and May 2018 yielding 96 raw prawns, 107 pre-cooked prawns and 97 pangasius fillets. The majority of samples (97%) were from Vietnam. Of the 300 samples, *Enterococcus faecalis* was detected in 87.0% (CI 95% 83;93), *E. faecium* in 21.7% (CI 95% 17;27) and *E. coli* in 22.3% (CI 95% 18;27). Both *E. faecalis* and *E. facium* were detected in 57 samples and *E.coli* was only detected in combination with enteroccci. Of the isolates, 65.7% (CI 95% 57;73) *E. faecalis*, 1.5% (CI 95% 0.9;10) *E. faecium* and 40.3% (CI 95% 29;52) of *E. coli* were fully sensitive to all antimicrobials in the panel tested. In 62 of the 300 samples (20.1% (CI 95% 16;26)), resistance to at least one of the critically important and highest priority antimicrobials as classified by WHO was detected. No resistance to carbapenem, vancomycin or linezolid was detected, but one *E. coli* isolate carried resistance genes to multiple antibiotics including cephalosporins, colistin, fluoroquinolones and macrolides.

Keywords: imported seafood, antimicrobial resistance, microbiological contamination, prawns, pangasius
1. Introduction

The emergence and increase of antimicrobial resistance (AMR) is an internationally recognized problem and “The FAO action plan on Antimicrobial Resistance 2016-2020” supports the agricultural and food industries in tackling AMR worldwide (FAO, 2016). The Food and Agriculture Organization of the United Nations (FAO) recognizes that the risk of increasing AMR appears to be higher in countries with weaker legislation and regulatory systems for use of antimicrobial drugs than countries with implemented action plans and surveillance on the use of antimicrobials (Hendriksen et al., 2019; Thornber et al., 2019). Global food trade is likely to play a role in spreading AMR between countries and well-regulated countries may be at risk of introducing novel resistant pathogens, resistance genes and increasing the national burden of AMR via imported foods.

In 2016, global aquaculture production was 110.2 million tonnes with a first-sale value estimated at USD 243.5 billion. Asian countries, in particular China are the main producers accounting for nearly 90% of the global production (FAO, 2018). Prawns and fish, including pangasius (*Pangasianodon hypophthalmus*), are main commodities produced and exported. Intensification of aquaculture in Asian countries has often been accompanied by a higher frequency of outbreaks of infectious bacterial diseases that require preventive and control measures, e.g. antimicrobial treatments. Whilst the use of antimicrobials may have benefited aquaculture production, it has also attracted some criticism due to negative environmental impacts and development of AMR among the bacterial populations in ponds and cultured aquatic species. Concerns that AMR genes, resistant bacteria and antimicrobial residues may be
transferred to humans through consumption of fish and prawns raised in aquaculture have been expressed (Watts et al., 2017).

Vietnam is the main global pangasius producer, but has experienced a fall in exports to the European Union (EU) during recent years (Globefish, 2018). The effects of weak consumer demand, damaging media coverage and strong competition from whitefish alternatives have now been compounded by the high price level on other import markets (Globefish, 2018). EU member countries continue to import large volumes of prawns, mainly vannamei prawns (Litopenaeus vannamei) from Asia, particularly from Vietnam (Globefish, 2018). However, the public perception of tropical farmed prawns and pangasius tends to be increasingly negative, perpetuated by negative mainstream and internet based media stories, blogs and information outlets (Little et al., 2012; Murk, Rietjens, & Bush, 2018). A recent assessment of the toxicological risks of consuming imported Asian prawns in the EU suggested a reduced consumption risk. This was mainly based on fewer RASFF (Rapid Alert System on Food and Feed) alerts than expected, when taken the increased supply over the lifetime of the alerts system into account (Newton, Zhang, Leaver, Murray, & Little, 2019). In contrast to the monitoring of antimicrobial residues, AMR in pangasius and prawns is not routinely monitored by the EU member countries. Such a monitoring is challenged by the lack of good indicators of AMR in seafood as the traditional bacterial indicators of antimicrobial resistance used in livestock meat types, i.e. *Escherichia coli* (*E. coli*) and *Enterococci faecium* (*E. faecium*) and *Enterococci faecalis* (*E. faecalis*), are not part of the normal microbiota in seafood. *E. coli* and enterococci have, however, been used to study the antimicrobial resistance from raw fish and seafood imported into Switzerland (Boss, Overesch, & Baumgartner, 2016). The
same authors furthermore proposed *E. coli* and *E. faecalis* in pangasius and shrimps as potential candidates for programs monitoring antimicrobial resistance. Dib et al., 2018, also found antimicrobial resistance in *E. coli* isolated from seafood in Constantine, Northeast Algeria.

The aim of this study was to investigate levels of AMR in *E. coli* and enterococci introduced to Denmark via prawns and pangasius products imported from Asia.

2. Materials and methods

2.1. Sample collection

In total, 300 samples of frozen pangasius (*Pangasianodon hypophthalmus*) fillets and prawns (Penaeidae family) imported from Asia were collected from retail shops around Denmark. Samples were collected every two months between September 2017 and May 2018 by the regional food officers from the Danish Veterinary and Food Administration (DVFA).

The number of shops, establishments and samples selected by each regional DVFA control unit was proportional to the number of establishments in the region relative to the total number of establishments in the country. Samples were equally distributed between pangasius, raw prawns and ready-to-eat prawns. Samples remained frozen until analysis and were analyzed before the expiry date of the product.

2.2. Laboratory methods

2.2.1. Detection of *E. coli* and enterococci

A total of 25 g thawed sample was added to 225 ml Buffered Peptone Water (BPW) in a sterile stomacher bag and homogenized for 30 sec. For detection of enterococci, 100 µl of the
suspension was streaked onto Slanetz and Bartley agar (Bio-rad, Denmark) and incubated at 41.5°C for 48 h. For detection of *E. coli*, 100 µl of the suspension was streaked onto Violet Red Bile (RVG) agar (Difco, Denmark) and incubated at 30°C for 4-6 hours followed by incubation at 44°C over night. The remaining suspension was incubated at 37°C for 18-22 h (hereafter referred to as overnight culture).

In case growth of enterococci or *E. coli* was not observed on the agar plates, 10 µl of the overnight culture was plated onto Slanetz Bartley and RVG agar again and incubated as described above without the incubation step at 30°C for *E. coli*.

Furthermore, to screen for the critically important cephalosporin- or carbapenem resistant *E. coli* the overnight culture was streaked on each of the following agar plates: 10 µl on MacConkey (MCA) agar containing 1 µg/ml of cefotaxime (Tritium, Netherlands) and 20 µl on ChromID CARBA and ChromID OXA-48 (Biomerioux, France). The MCA and ChromID agar plates were incubated at 44°C for 18-22 h and 35-37°C for 18-24 h, respectively.

From each agar plate, up to three colonies resembling *E. coli*, two colonies resembling *E. faecalis* and another two resembling *E. faecium* were selected for species verification. One isolate of presumptive cephalosporin- and carbapenem resistant *E. coli* was selected per sample.
2.2.2. Species verification

Presumptive *E. coli* colonies were verified on Tryptone Bile Glucuronic (TBX) agar (Oxoid, Denmark) incubated at 44°C overnight. Presumptive enterococci colonies were sub-cultivated on blood agar and identified by a real-time PCR assay (Dutka-Malen, Evers, & Courvalin, 1995). All verified isolates were stored at –80°C until further analysis.

2.2.3. AMR identification

Antimicrobial susceptibility testing (AST) was performed by Minimum Inhibitory Concentration (MIC) determination using broth microdilution (Sensititre, Trek Diagnostic Systems Ltd.). The antimicrobial panels and interpretive criteria used were in accordance with the Decision 2013/652/EU on the EU harmonized monitoring of AMR in foodborne bacteria. All procedures were performed according to ISO 20776-1:2006 standard. AST was performed for one isolate per sample for each bacterial species.

WGS and bioinformatics tools were used to characterize carbapenem and cephalosporin-resistant *E. coli* as confirmed by AST. Genomic DNA (Easy-DNA, Invitrogen, Carlsbad, CA, USA) was prepared for paired-end sequencing, 2x251 cycles, on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA). Library preparation was performed with the present version of the NexteraXT® protocol (Guide 15031942, Illumina, Inc., San Diego, CA). Raw sequence data were *de novo* assembled using SPAdes (Bankevich et al., 2012), and assembled sequences were analyzed using the Centre for Genomic Epidemiology (www.genomicepidemiology.org) tools including MLST Finder 2.0 (Larsen et al., 2012) for multi locus sequence typing (MLST) and
ResFinder 3.1 for detection of genes and chromosomal mutations mediating AMR (E. Zankari et al., 2012; Ea Zankari et al., 2017).

2.2.4. Data cleaning and analysis

Data was extracted from the DVFA laboratory information management system (LIMS) and validated in Microsoft Excel 2016. Further data cleaning, formatting and analysis were performed in STATA 14 (Statacorp, Texas, USA).

Data was tabulated, examined for outliers and described using Wilson Score Interval confidence intervals. Univariate comparisons by $\chi^2$ tests and logistic regression analyses adjusted for confounding, when appropriate.

3. Results

In total, 97 frozen, raw pangasius fillets were sampled and analyzed for *E. coli* and enterococci (Table 1). All pangasius fillets originated from Vietnam and three were repacked after arrival in Denmark. The majority of pangasius were raised in aquaculture with only two products of wild-caught pangasius.

Of the 203 frozen prawn samples, 107 were cooked and 96 were raw products. The majority of the cooked prawns were pre-peeled (65%) or partially peeled with only the tail-shell remaining (13%), whereas most of the raw prawns products were shell-on (81%). Of the pre-peeled or partially peeled prawns, only five samples had visible remains of the intestinal tract. The majority of the prawn products were from Vietnam (n=194), followed by Bangladesh and India (four from each) and one sample’s origin was described as from “Indonesia, Vietnam or
“Ecuador”. The majority of the products were packed in their country of origin. However, 13 products were re-packed in Denmark, three in France and one in the Netherlands. Almost all prawns were farmed (97%).

Only 31 of the 300 pangasius and prawn samples were negative for both *E. coli* and enterococci. *Enterococcus faecalis* was detected in 87.0% (CI$_{95\%}$ 83;93) of the samples, *E. faecium* in 21.7% (CI$_{95\%}$ 17;27) and *E. coli* in 22.3% (CI$_{95\%}$ 18;27) of samples. Both *E. faecalis* and *E. facium* were detected in 57 samples and *E. coli* was only detected in combination with enterococci.

### 3.1. *Enterococci spp.*

The majority of samples (89.7%, CI$_{95\%}$ 86;93) were contaminated by at least one of the two *Enterococcus* spp. and at least one *Enterococcus* spp. was isolated from all pangasius fillets and 84% of the prawns. Raw prawns were significantly more likely to be contaminated by enterococci than cooked prawns (93.8% vs. 76.6%, $p\leq 0.001$). Shell-on prawns were also more likely to contain enterococci, but this association was confounded by the fact that most cooked prawns were pre-peeled and the risk associated with shell-on prawns disappeared once adjusted for whether or not they were cooked.

Both *E. faecalis* and *E. facium* were detected in 57 samples (five pangasius and 52 prawn samples), whereas *E. faecalis* was detected alone in 204 samples and *E. faecium* in eight samples. Both species were detected in 18.7% of the cooked prawns and in 33.3% of the raw prawn products. Detection of enterococci species was independent of the visibility/presence of the intestinal tract.
A total of 140 *E. faecalis* and 65 *E. faecium* isolates were selected for MIC testing (Fig. 1). The majority of *E. faecalis* (65.7%, CI 95% 57;73) were sensitive to all antimicrobials tested, 27.9% (CI 95% 21;36) were resistant to one antimicrobial, 3.6% (CI 95% 1.5;8.1) to two antimicrobials and four strains (2.9%, CI 95% 1.1;7.1) were resistant to three or more of the antimicrobials in the panel (MDR). All multi-resistant strains were resistant to chloramphenicol, erythromycin, and tetracycline and one strain was further resistant to gentamicin. *E. faecalis* is intrinsically (i.e. naturally) resistant to streptogramin A and B (quinupristin-dalfopristin), and interpretation of the MIC testing for this drug was therefore not evaluated.

Only 1.5% (CI 95% 0.9;10) of *E. faecium* isolates were susceptible to all antimicrobials and 20% (CI 95% 12;31) of the isolates were resistant to three or more antimicrobials in the panel (MDR). All the MDR isolates were resistant to erythromycin, quinopristin-dalfopristin and tetracycline and two strains were additionally resistant to either ciprofloxacin or chloramphenicol.

No resistance to the last-line critically important drugs: linezolid or vancomycin was detected in any of the enterococci isolates by the methods applied, suggesting that resistance to these drugs would be present in less than 1% of imported seafood from Asia.

### 3.2. *E. coli*

*E. coli* was detected in 67 samples on agar plates without antimicrobial agents and was significantly more often detected in pangasius fillets (74.6%) than in prawns (25.4%) (p<0.001). The majority of contaminated prawn samples were raw, but *E. coli* was also detected in two cooked samples originating from Bangladesh and Vietnam, respectively. Every *E. coli* contaminated sample was also contaminated with enterococci.
Standard panel antimicrobial testing was performed for all 67 isolates. A total of 40.3% (CI 214 29;52) were sensitive to all antimicrobials tested. The levels of resistance to different antibiotics based on MIC are shown in Fig. 2. Only one strain isolated on non-selective media was resistant to cephalosporins (cefepime, ceftazidime and cefotaxime).

Ten isolates were resistant to three or more antimicrobial groups. The MDR isolates displayed various profiles (Table 2) with co-resistance to ampicillin, ciprofloxacin, tetracycline and trimethoprim being observed in the majority (60%) of the MDR isolates in combination with resistance to additional compounds. No resistance to nalidixic acid was detected in the MDR isolates, despite nearly all of them exhibiting ciprofloxacin resistance.

3.3. Screening for cephalosporin- and carbapenem resistant E. coli

Four E. coli were isolated on cefotaxime-containing agar plates whereas no E. coli were isolated on agar plates selective for carbapenemase-producers. These E. coli were isolated from prawn samples only, three samples from Vietnam and one from Bangladesh. WGS data showed that these isolates displayed different STs and harboured two different Extended Spectrum Beta-Lactamase (ESBL)-encoding genes, namely $bla_{CTX-M-15}$ or $bla_{CTX-M-55}$ (Table 3). Furthermore, all isolates harboured genes conferring resistance to additional antimicrobials including the plasmid-mediated quinolone resistance (PMQR) gene $qnrS1$ and, notably, the colistin resistance gene $mcr-1$ occurring in one Vietnamese, shell-on, raw prawn.

3.4. Overall resistance imported via the products

In 62 of the 300 samples (20.7% (CI 17;26), we detected resistance to at least one of the critically important and highest priority antimicrobials as classified by WHO (WHO, 2017).
4. Discussion

The majority of samples originated from Vietnam (97.0%), which probably reflects the origin of Asian prawns and pangasius in the Danish retail stores. We found enterococci and/or *E. coli* in a large proportion (89.7%) of the seafood samples. This was surprising, especially in the pre-cooked prawns, where 71.7% of the samples were contaminated with either enterococci or *E. coli*. This suggests that the contamination occurred late in the processing after cooking. Very often Northern Europeans consider cooked prawns ready-to-eat and consumer advice may be considered appropriate. The fact that so many samples contained detectable levels of enterococci and *E. coli* even after freezing also suggested that the initial contamination levels were very high. This means that pathogens may survive too and the products may pose a risk to consumers.

A considerable proportion (45.6%) of enterococci displayed resistance to at least one antimicrobial. However, occurrence of AMR differed considerably between the two enterococci species analyzed, and results were in line with the notion that acquired AMR occurs more frequently in *E. faecium* than in *E. faecalis* (Hollenbeck & Rice, 2012).

We found very high occurrence of tetracycline and quinupristin-dalfopristin resistance in *E. faecium* and tetracycline resistance in *E. faecalis*, which is similar to other food matrices in other EU countries (Danmap, 2017). Tetracycline and similar antimicrobial products were used daily in Vietnam until the national action plan was implemented in 2016, which may explain the high resistance levels to tetracycline (Long and Lua, 2017).
Of the *E. coli* isolates obtained on agar plates without antimicrobial agents, 40.2% were resistant to at least one antimicrobial. Very high occurrence of ciprofloxacin resistance was detected, despite a ban on using fluoroquinolones in Vietnamese aquaculture was instated in 2016 (Long and Lua, 2017). The phenotypic resistance was often detected in the presence of nalidixic acid susceptibility, which is a phenotype generally mediated by PMQR genes. This suggests that PMQR genes were circulating widely in *E. coli* in Asian and Vietnamese seafood imported to Denmark at the time of sampling. Although the public health significance of PMQR genes has not yet been fully elucidated, it is known that occurrence of such genes facilitates the selection of high level of resistance to quinolones (Poirel, Cattoir, & Nordmann, 2012), which are among the highest priority critically important antimicrobials for human medicine. This phenotype is not common in Danish food and imports may be a source of PMQR genes for humans (Danmap, 2018).

On a positive note, only very low occurrence of resistance to other highest priority critically important antimicrobials such as third-generation cephalosporins and colistin was detected. No resistance to carbapenems or macrolides was observed. Carbepenem resistance was detected in seafood imported from Southeast Asia to Canada suggesting food as a potential source of exposure to consumers (Janecko et al., 2016). When analyzing the same samples by a selective culture procedure, only a low proportion (1.3%) yielded third-generation cephalosporin-resistant *E. coli* indicating that overall this phenotype occurred sporadically in *E. coli* in Asian seafood imported to Denmark at the time of sampling. The third-generation cephalosporin resistance was mediated by ESBL-encoding genes such as *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-55}$ that have been commonly described in bacteria from human and animal sources in Vietnam and other
Asian countries (Bui et al., 2015; Hoang et al., 2017; Nguyen et al., 2016; Zurfluh et al., 2015).

These ESBLs have also been described sporadically in isolates from Danish production animals and retail meat of Danish origin whereas they are among the most commonly detected ESBLs in human clinical isolated in Denmark (Danmap, 2018).

One *E. coli* isolate harboured a $\text{bla}_{\text{CTX-M-55}}$-gene and was resistant to antimicrobials from nine classes, including four classified as highest priority critically important antimicrobials for human medicine (Table 3, isolate 4). Most of the AMR genes detected in this isolate have been previously described on mobile genetic elements, and although we did not verify if these genes were transferrable to other bacteria, it is of high concern that an *E. coli* resistant to virtually all antimicrobials available for therapy occurs in food.

### 5. Conclusions

In this study, only one MDR isolate from a raw prawn sample from Vietnam harboured the many very rare resistance genes. Nonetheless, in combination with the high proportion of contaminated pre-cooked samples, high number of mobile resistance genes present and the Danish habit of eating cold pre-cooked prawns, even 1 in 300 imported seafood samples could expose consumers in Denmark to AMR genes that are very rare in domestic food sources, such as plasmid-mediated fluoro-quinolones or carbapenem.

**Acknowledgement**

The survey was funded by the Danish Veterinary and Food Administration, who also sampled and carried out the laboratory analysis.
Conflict of interest

Declarations of interest: none

References


Legends

Tables:

Table 1. Description of 300 retail samples of pangasius (*Pangasianodon hypophthalmus*) fillets
and prawns (Penaeidae family) from Asia collected in Danish supermarkets.

Table 2. Resistance profiles for 10 MDR *E. coli* isolates.

Table 3. Phenotypic and genotypic traits of four ESBL-producing *E. coli* isolates from Asian
prawns recovered from the overnight culture.

Figures:

Figure 1. Resistance to different antimicrobial agents in 140 *E. faecalis* and 65 *E. faecium*
originating from pangasius and prawns from Asia. *E. faecalis* is intrinsic resistant to
quinupristin/dalfopristin and therefore not shown.

Figure 2. Proportion of antimicrobial resistance in the 67 *E. coli* strains isolated from pangasius
and prawn samples.
Table 1.

<table>
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<th></th>
<th>Total</th>
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<td>17</td>
<td>172</td>
<td>31</td>
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<td>0</td>
</tr>
<tr>
<td>Bangladesh</td>
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<td>4</td>
<td>0</td>
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<tr>
<td>Other**</td>
<td>1</td>
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<td>Prawns (n=203)</td>
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<tr>
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<td>13</td>
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<td>Wild-caught prawns</td>
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<td>0</td>
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*E. coli obtained on agar plates without antimicrobial agents

**Origin of sample “Indonesia, Vietnam or Ecuador”
Table 2.

<table>
<thead>
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<th>MDR pattern</th>
<th>Number of strains</th>
</tr>
</thead>
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<td>SMX; TET; TMP</td>
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</tr>
<tr>
<td>CIP; TET; TMP</td>
<td>3</td>
</tr>
<tr>
<td>AMP; CIP; SMX; TET; TMP</td>
<td>2</td>
</tr>
<tr>
<td>AMP; CHL; CIP; SMX; TET; TMP</td>
<td>3</td>
</tr>
<tr>
<td>AMP; CIP; CHL; FEP; FOT; TET; TMP; TAZ</td>
<td>1</td>
</tr>
<tr>
<td>Total MDR</td>
<td>10</td>
</tr>
</tbody>
</table>

Note: AMP=Ampicillin; CIP=Ciprofloxacin; CHL=Chloramphenicol; FEP=Cefepime; FOT=Cefotaxime; SMX=Sulphamethoxazole; TAZ=Ceftazidime; TET=Tetracycline; TMP=Trimethoprim; MDR=Multi drug resistance
<table>
<thead>
<tr>
<th>Isolate number</th>
<th>ST</th>
<th>Phenotypic resistance profile</th>
<th>ESBL genes (WGS and Resfinder 3.1)</th>
<th>Additional AMR genes</th>
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<td>AMP CIP FEP FOT TAZ</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>qnrS1</td>
</tr>
<tr>
<td>2</td>
<td>ST226</td>
<td>AMP CIP FEP FOT TAZ</td>
<td>bla&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>qnrS1</td>
</tr>
<tr>
<td>3</td>
<td>Unknown</td>
<td>AMP CHL CIP FEP FOT TAZ TET TMP</td>
<td>bla&lt;sub&gt;CTX-M-55&lt;/sub&gt;</td>
<td>aadA5, bla&lt;sub&gt;TEM-18&lt;/sub&gt;, dfrA17, floR, qnrS1, tet(A)</td>
</tr>
<tr>
<td>4</td>
<td>Unknown</td>
<td>AMP AZI CHL CIP COL FEP FOT FOX GEN NAL SMX TAZ TET TMP</td>
<td>bla&lt;sub&gt;CTX-M-55&lt;/sub&gt;</td>
<td>aac(3)-IId, aadA22, aadA5, aph(3’)-Ia, aph(6)-IId, dfrA17, mcr-1, mph(A), Inu(F), FloR, GyrA S83L*, qnrS1, Sul2, sul3, tet(A)</td>
</tr>
</tbody>
</table>

Note: AMP=Ampicillin; AZI=Azithromycin; CIP=Ciprofloxacin; CHL=Chloramphenicol; COL=Colistin; FEP=Cefepime; FOT=Cefotaxime; FOX=Cefoxitin; GEN=Gentamicin; NAL=Nalidixan; SMX=Sulphamethoxazole; TAZ=Ceftazidime; TET=Tetracycline; TMP=Trimethoprim; MDR=Multi drug resistance. Isolates discovered by selective enrichment methods. *Mutational resistance.
Figure 1.
Figure 2.
Highlights

• Samples of frozen pangasius fillets and prawns imported from Asia to Denmark were analysed.
• The majority of samples were contaminated by at least one of the two *Enterococcus* spp.
• Resistance to critically important and highest priority antimicrobials were detected.
Declarations of interest: none