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Original scientific paper

Detection of pathogenic Yersinia enterocolitica strains in pre-packed fresh pork minced meat — preliminary data

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ABSTRACT

Yersinia plays an important role as the third most common zoonotic agent of human gastrointestinal diseases in the European Union (EU). Most frequently, an infection in humans is caused by *Yersinia (Y.) enterocolitica*, whereas *Y. pseudotuberculosis* is mainly pathogenic for animals and less frequently associated as a causative agent of foodborne yersiniosis. Pigs are common carriers of *Y. enterocolitica*, which can lead to contamination of pork meat during the slaughter process. In our study, we investigated the occurrence of pathogenic *Y. enterocolitica* strains in pre-packed fresh pork minced meat from supermarkets in Southern Berlin and Brandenburg. In 17 of 104 samples *Y. enterocolitica* was detected by applying two ISO methods (ISO 10273:2017 and CEN ISO/TS 18867). With Illumina short read sequencing technology, an initial insight into genomic properties of selected *Y. enterocolitica* strains was obtained. Further characterisation and sequencing of additional *Y. enterocolitica* strains obtained within this study is planned.

1. Introduction

According to European Food Safety Authority (EFSA), yersiniosis was the third most commonly reported bacterial zoonosis, with 6,789 confirmed human cases, reported by 26 member states (MS) in 2021. The EU notification rate was 1.9 cases per 100,000 population, with an increase of 11.8% compared to 2020 (1.7 per 100,000 population) (*EFSA and ECDC*, 2022). As for Germany, 1,873 cases were reported in 2020. This corresponded to an incidence of 2.3 cases per 100,000 population. The majority of these cases (99%) were caused by *Yersinia (Y.) enterocolitica*, predominantly by the bio/serotype 4/ O:3 (82%) (RKI, 2020).

Pigs are commonly hosts of *Y. enterocolitica*, and this can lead to the contamination of raw pork meat during the slaughter and processing process. A study from Germany in 2018 revealed that among 253 samples of raw minced pork meat and pork meat preparations, *Y. enterocolitica* was detected in approximately one of ten samples (9.5%) (*Verbraucherschutz Sachsen-Anhalt*, 2018). On the other hand, *Y. pseudotuberculosis* is frequently associated with its reservoirs, birds and wild animals (*BfR*, 2013). Outbreaks with *Y. pseudotuberculosis* are mostly linked to raw vegetables and ready-to-eat vegetable products, such as lettuce and carrots, with long periods of cold storage. Although *Y. enterocolitica* is often associated with pork meat, an increasing number of outbreaks are also sporadically linked to vegetables (*ECDC*, 2022). Each of these two *Yersinia* species plays an important role as the causative

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agent of gastrointestinal yersiniosis. Since *Yersinia* can multiply even at 4°C, it is important to reduce the food contamination significantly. The bacteria are mainly found in the tonsils, lymph nodes and intestines of pigs. As pigs are frequently asymptomatic carriers, contamination during the slaughter process must be avoided as much as possible (*Fredriksson-Ahomaa*, 2012; *BfR*, 2013). In humans, symptoms such as fever and abdominal pain in the right lower abdomen can appear after an incubation period of 4–10 days. In children, bloody diarrhoea can occur as an additional symptom (*EFSA and ECDC*, 2022).

Within the species *Y. enterocolitica*, six biovars are distinguished (1A, 1B, 2, 3, 4, 5). Strains belonging to biovar 1B to 5 possess a virulence plasmid (pYV) and virulence factors, such as enterotoxin YstA, and are considered pathogenic (obligate human pathogen). The biovar 1A strains lack the virulence plasmid; however, they possess the heat-stable enterotoxin (YstB), relevant to the development of diarrhoea (*RKI*, 2021). Although it is assumed that biovar 1A is non-pathogenic, there are indications that certain 1A strains can cause gastrointestinal symptoms as observed in pYV-bearing strains (*Tennant et al*., 2003). Biovar 4 (serotype O:3) and biovar 2 (serotype O:9) are most frequently associated with human yersiniosis in Europe.

In our study, we investigated the occurrence of pathogenic *Y. enterocolitica* strains in 104 samples of pre-packed fresh pork minced meat by applying two ISO methods (ISO 10273:2017 (*ISO*, 2017) and CEN ISO/TS 18867 (*ISO*, 2015). Using Illumina NGS sequencing, we gained initial insight into the genomic properties (such as multilocus sequence typing (MLST) types, antimicrobial resistance (AMR) and virulence genes) in a selection of *Y. enterocolitica* strains. Further serovar and genomic characterisation of current and additional *Y. enterocolitica* strains obtained within this study will follow.

2. Materials and methods

From May to December 2021, in total, 104 pre-packed fresh pork minced meat packages, ranging in weight from 250 grams (g) to 1 kilogram (kg), were purchased in 11 different supermarkets in Southern Berlin and Brandenburg. The samples were transported chilled and submitted to microbiological analysis within the same day. Isolated *Y. enterocolitica* strains were preserved in cryoprotective medium at –80°C for later molecular analysis.

2.1 Isolation and biotyping of Y. enterocolitica strains

The isolation and biotyping of pathogenic *Y. enterocolitica* strains was conducted according to *ISO* (2017). Briefly, 25 g of sample was tenfold diluted in PSB-Bouillon (Peptone Sorbitol Bile Broth) and 1 (mL) was plated directly onto 4 CIN (Cefsulodin-Irgasan-Novobiocin) agar plates and incubated at 30°C for 24 ± 2 hours (h). Additionally, 10 mL were added to 90 mL of ITC (Irgasan-Ticarcillin-Chlorat-Bouillon) as a second enrichment medium. Both enrichment broths were incubated at 25°C for 44 h ± 4 h. From these two enrichment media, 0.5 mL were added to 4.5 mL of 0.5% potassium hydroxide (KOH) for 20 ± *5* seconds (sec.). After the treatment, the CIN agar plates were inoculated and incubated as previously described. The CIN agar plates were investigated under a magnifier to detect characteristic bull's eye colonies of *Y. enterocolitica*. Characteristic colonies were biotyped by testing their reaction to aesculin/salicin, xylose, pyrazinamidase, tween-esterase/lipase, trehalose and indole.

For further confirmation, the MALDI-TOF system was used by transferring a small proportion of the suspect colony from TSA (Tryptic Soy Agar) directly onto a MALDI-TOF target plate and adding 1 microliter (μL) of HCCA matrix. Further steps and the use software were conducted according to the manufacturer's instructions.

To investigate the pathogenic potential of the isolated *Y. enterocolitica* strains, thermal lysis of the selected strains followed by real time PCR, targeting a sequence of the *ail* gen according to CEN ISO/TS 18867 (*ISO*, 2015), was conducted. For specific details on real time PCR, please refer to CEN ISO/TS 18867.

2.2 Illumina whole genome sequencing

One colony of presumptive Y. *enterocolitica* strain was inoculated into 4 mL of Luria Bertani Broth and incubated overnight while gently shaking at 37°C. The following day, the suspension was centrifuged at 13,000 rpm for 5 minutes, and the bacterial pellet was stored at −20°C for the later DNA isolation.

The genomic DNA was extracted using Purelink Genomic DNA Kit according to the manufacturer's instructions. The ILMN DNA LP (M) Tagmentation Kit (96Samples) Beads + Buffers were used for preparing the Illumina sequencing library

Table 1. MLST, AMR and virulence genes of selected *Y. enterocolitica* strains

Strain ID	Total length (bp)	MLST	AMR genes	Some virulence genes
LM00140	4,480,285	135	*bla*A,*vat*(F)	*yst*A, *vir*F, *ysc*, *yop*
LM00141	4,474,096	135	*bla*A,*vat*(F)	*yst*A, *vir*F, *ysc*, *yop*
LM00150	4,437,788	135	*bla*A,*vat*(F)	*yst*A, *vir*F, *ysc*, *yop*
LM00196	4,480,780	135	*bla*A,*vat*(F)	*yst*A, *vir*F, *ysc*, *yop*
LM00921	4,457,321	135	*bla*A,*vat*(F)	*yst*A, *vir*F, *ysc*, *yop*
LM00930	4,475,386	135	*bla*A,*vat*(F)	*yst*A, *vir*F, *ysc*, *yop*

with Nextera™ DNA CD Indexes (96 Indexes, 96 Samples). Sequencing was carried out in 2×151 bp cycles on an Illumina MiSeq system using MiSeq Reagent Kit v3 (600-cycle).

The assembly and quality assessment of microbial isolate sequencing experiments were conducted by AQUAMIS (*Deneke et al*., 2021). Further characterisation of the bacterial genomes was conducted by BakCharak, yielding insight AMR genes, plasmids and virulence factors.

3. Results

Pathogenic *Y. enterocolitica* strains were detected in 17 out of 104 pre-packed fresh pork minced meat samples. The typical bull's eye colonies on CIN agar plates were submitted to thermal lysis, and the DNA was tested by real time PCR (according to *ISO*, (2015), i.e., CEN ISO/TS 18867) and yielded a positive signal, indicating potential pathogenic properties of these *Y. enterocolitica* strains. Two positive minced meat samples had an identical batch number and were sampled on two consecutive days in the same supermarket. In three samples, the typical bull's eye colonies could be quantified (10–20 CFU/g sample) after direct plating on CIN agar. The remaining strains could only be isolated after enrichment in PSB and/or ITC according to *ISO* (2017). Strains selected for further sequencing by Illumina short read technology are shown in Table 1, along with certain outputs of the Aquamis and BakCharak pipelines. For further details on more virulence genes and genes associated with metal resistance, please refer to the authors.

The biotyping according to *ISO* (2017) revealed that the six tested *Y. enterocolitica* strains belong to biovar 4. Serotyping of the obtained isolates was not conducted.

4. Discussion

As in previous years, yersiniosis still plays an important role as a human gastrointestinal disease in the European Union (EU). Similar to other foodborne associated zoonotic bacteria, it is especially important to prevent infections in children, older and immunocompromised people. Children under five years of age are most commonly affected by yersiniosis. According to *RKI* (2020), in Germany, the majority of cases (about 98%) occurs as sporadic cases. A study by *Rosner et al.* (2012) observed that the consumption of raw minced pork, which is also frequently consumed by young children in Germany, was the main risk factor for the disease in Germany. Another risk factor was the preparation of minced pork in the household. These findings confirm that the common route of infection is the consumption of raw or undercooked pork, e.g. as ground pork or minced pork, and hygiene deficiencies in the preparation of ground pork in the household.

Our study showed that pre-packed fresh pork minced meat can be contaminated with *Y. enterocolitica.* It is, therefore, particularly important to avoid the consumption of raw or undercooked pork and to apply appropriate hygienic measures when handling raw pork meat. Proper heating of meat for at least 2 minutes at 70°C is deemed sufficient to kill *Yersinia*, provided that that this temperature is also reached in the core of the food (*BfR*, 2012).

Further characterisation and sequencing of the remaining *Y. enterocolitica* strains obtained in this study will continue for genome characterisation and to determine the degree of their genetic diversity. The detection of particular AMR genes should be supported by respective phenotypical tests to investigate phenotypical antimicrobial resistance in these *Y*. enterocolitica strains.

5. Conclusion

Since pre-packed fresh pork minced meat can be contaminated with pathogenic *Y. enterocolitica* strains, it is critical to avoid eating raw or undercooked pork, e.g., in the form of ground pork or minced pork. Furthermore, appropriate hygiene measures when handling raw pork are crucial to prevent cross-contamination with other foods and to minimise the potential risk of infection for consumers. This applies all the more to households with small children or otherwise vulnerable groups of people.

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