



Prevalence and characterization of ESBL- and AmpC-producing Enterobacteriaceae on retail vegetables

Angela H.A.M. van Hoek^{a,*}, Christiaan Veenman^a, Wendy M. van Overbeek^a, Gretta Lynch^a, Ana Maria de Roda Husman^{a,b}, Hetty Blaak^a

^a Centre for Infectious Disease Control (CIb), National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands

^b Faculty of Veterinary Medicine, Institute for Risk Assessment Sciences (IRAS), Utrecht University, Utrecht, The Netherlands

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ABSTRACT

In total 1216 vegetables obtained from Dutch stores during 2012 and 2013 were analysed to determine the prevalence of 3rd-generation cephalosporin (3GC) resistant bacteria on soil-grown fresh produce possibly consumed raw. Vegetables grown conventionally and organically, from Dutch as well as foreign origin were compared. Included were the following vegetable types; blanched celery ($n = 192$), bunched carrots ($n = 190$), butterhead lettuce ($n = 137$), chicory ($n = 96$), endive ($n = 188$), iceberg lettuce ($n = 193$) and radish ($n = 120$). Overall, 3GC-resistant Enterobacteriaceae were detected on 5.2% of vegetables. Based on primary habitat and mechanism of 3GC-resistance, these bacteria could be divided into four groups: ESBL-producing faecal species (*Escherichia coli*, *Enterobacter* spp.), AmpC-producing faecal species (*Citrobacter freundii*, *Enterobacter* spp.), ESBL-producing environmental species (*Pantoea* spp., *Rahnella aquatilis*, *Serratia fonticola*), and AmpC-producing environmental species (*Cedecca* spp., *Hafnia alvei*, *Pantoea* spp., *Serratia plymuthica*), which were detected on 0.8%, 1.2%, 2.6% and 0.4% of the vegetables analysed, respectively. Contamination with faecal 3GC-resistant bacteria was most frequently observed in root and bulb vegetables (average prevalence 4.4%), and less frequently in stem vegetables (prevalence 1.6%) and leafy greens (average prevalence 0.6%). In Dutch stores, only four of the included vegetable types (blanched celery, bunched carrots, endive, iceberg lettuce) were available in all four possible variants: Dutch/conventional, Dutch/organic, foreign/conventional, foreign/organic. With respect to these vegetable types, no statistically significant difference was observed in prevalence of 3GC-resistant Enterobacteriaceae between country of origin or cultivation type (5.2%, 5.7%, 5.7% and 3.3%, respectively). Vegetables consumed raw may be a source of dissemination of 3GC-resistant Enterobacteriaceae and their resistance genes to humans. The magnitude of the associated public health risk presumably depends on the types of bacteria that are ingested, i.e., faecal or environmental species, and may therefore be higher for root and bulb vegetables compared to leafy greens.

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1. Introduction

Fresh produce is considered a good source of nutrients; consequently the consumption of fresh produce has been increasing as ingredients of healthy diets in the last decade (Olaimat and Holley, 2012). Because vegetables are often consumed raw, consumption may result in the ingestion of microorganisms which pose a potential risk to consumer health (Franz et al., 2008; Heaton and Jones, 2008). Foodborne outbreaks have been associated with consumption of fresh produce (Lynch et al., 2009; Tyler and Triplett, 2008). In addition, vegetables represent a route of human exposure to antibiotic resistant bacteria (Schwaiger et al., 2011; Walsh and Fanning, 2008). One of the major

concerns, regarding antibiotic resistance worldwide, is the dissemination of Gram-negative bacteria especially Enterobacteriaceae displaying resistance to 3rd generation cephalosporins (3GCs) (Cantón et al., 2008; Carattoli, 2008; Coque et al., 2008; Ewers et al., 2012; Jacoby, 2009). 3GCs are crucial antimicrobial agents in the defence against bacterial infections caused by Gram-negative bacteria. Resistance to 3GCs is generally mediated by the production of extended spectrum β -lactamases (ESBLs) or AmpC β -lactamases, and often associated with faecal Enterobacteriaceae such as *Citrobacter* spp., *Enterobacter* spp., *Escherichia coli*, and *Klebsiella* spp. These bacteria may end up in agricultural soil through the use of animal manure or faecal contaminated surface water for irrigation. Enterobacteriaceae such as *Kluyvera*, *Serratia* and *Rahnella* species with soil and water as primary habitat, are natural carriers of ESBL genes, and an additional source of 3GC-resistance determinants in the environment.

During cultivation in (manure-amended) soil and/or when irrigated with contaminated water, fresh produce may acquire ESBL- and AmpC-

* Corresponding author at: National Institute for Public Health and the Environment (RIVM) – CIb, Antonie van Leeuwenhoeklaan 9, NL-3721 MA Bilthoven, The Netherlands. Tel.: +31 30 2747058.

E-mail address: angela.van.hoek@rivm.nl (A.H.A.M. van Hoek).

producing bacteria from their environment. Especially raw consumption of fresh produce may result in the ingestion of 3GC-resistant bacteria that are able to colonize the gut and to exchange resistance genes with intestinal bacteria during passage through the intestines, thus posing a potential public health risk (Djordjevic et al., 2013; Smet et al., 2011). Only a few studies have addressed the presence of 3GC-producing Enterobacteriaceae on vegetables (Blaak et al., 2014; Egea et al., 2011; Hassan et al., 2011; Mesa et al., 2006; Raphael et al., 2011; Reuland et al., 2014; Ruimy et al., 2010; Schwaiger et al., 2011). In a recent study, 75 crops of iceberg lettuce obtained directly from farms at harvest and nearly 150 items of different types of retail vegetables all originating from Dutch farms were investigated (Blaak et al., 2014). In that study, both class A ESBL- (as defined by Ambler, 1980; Bush and Jacoby, 2010) and AmpC-producing Enterobacteriaceae were detected on fresh produce. However, all ESBL-producing isolates identified were environmental species, mainly *Rahnella aquatilis* and *Serratia fonticola*, whereas none of the detected 3GC-resistant *Citrobacter* spp. and *Enterobacter* spp. produced ESBL. Isolation of 3GC-resistant Enterobacteriaceae appeared severely hampered by the abundance of resistant background flora, mainly consisting of *Pseudomonas* spp., but also *Aeromonas* spp., *Acinetobacter* spp. and *Stenotrophomonas* spp. (Blaak et al., 2014). The main goal of the present study was to determine the prevalence of ESBL- and AmpC-producing Enterobacteriaceae on retail fresh produce in The Netherlands, by using an alternative, more sensitive isolation method and analysis of a large number of vegetables. The alternative isolation method entailed anaerobic culture conditions to reduce the growth of 3GC-resistant background species and the addition of an AmpC-inhibitor (phenylboronic acid) to enhance detection of ESBL-producers. Overall, 1216 vegetable items were investigated. Vegetable types were selected that are consumed raw (from a public health perspective), grown in soil and/or regularly irrigated because of cultivation in greenhouses (with a risk of environmental contamination); i.e., blanched celery, bunched carrots, butterhead lettuce, endive, iceberg lettuce, radish, and spring onion. Chicory was also analysed, even though the end product, the heads, are generally grown on water and only the roots are grown in soil. Prevalence was compared for conventional and organic grown vegetables as well as for Dutch and imported items.

2. Materials and methods

2.1. Fresh produce sampling

During the period of 27th March 2012 to 8th July 2013 conventional and organic vegetables were obtained from eight large store chain supermarkets and five organic green grocery shops in and around

Bilthoven, The Netherlands, divided over 19 (roughly 1-monthly) visits. In total 1216 items of blanched celery (*Apium graveolens* L. var. *dulce*), bunched carrots (*Daucus carota* L. subsp. *sativus*), butterhead lettuce (*Lactuca sativa* L. var. *capitata* L.), chicory (*Cichorium intybus* L.), endive (*Cichorium endivia*), iceberg lettuce (*L. sativa* L. var. *capitata* L.), spring onion (*Allium fistulosum*) and radish (*Raphanus sativus* L. var. *radicula*) were purchased (Table 1). For each vegetable type, organic and conventionally grown items were obtained, as well as Dutch (NL) and imported (non-NL) ones. The initial goal was 48 items of each combination of vegetable type/cultivation type/country of origin (NL vs. non-NL), however due to limited availability or even absence of specific combinations in Dutch stores, this was not always achieved (Table 1). The selection of vegetables from foreign countries was solely based on what was available in the stores at the time of the visit (i.e., on import policies), and not aimed to specifically include certain countries. If at any time-point items of the same vegetable type from different countries were available in one store, both were purchased. Samples were stored at 4 °C and analysed within 24 h after purchase.

2.2. Sample analysis

The vegetables were not rinsed prior to processing to represent a worst-case scenario, only brown leaves were removed. Leafy vegetables were peeled off aseptically and torn into smaller pieces, and the others were sliced into small chunks. To 60 g of vegetable pieces or chunks, 60 ml BPW (bioTRADING Benelux B.V., Mijdrecht, The Netherlands) was added. Next, the sample was homogenised for 1 min in a Stomacher® 400 Circulator (Seward, Ltd., Worthing, UK). From the thus obtained homogenates 2 and 20 ml (i.e., equivalents of 1 g and 10 g of vegetable extract) were incubated in a 10-fold volume of BPW (bioTRADING Benelux B.V., Mijdrecht, The Netherlands) supplemented with cefotaxime to a final concentration of 1 µg/ml (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 20 h ± 2 h at 37 °C. The selected cefotaxime concentration represented the epidemiological cut-off (ECOFF) value (i.e., the upper limit of wild-type sensitivity) for cefotaxime for *Citrobacter* spp. and *Enterobacter* spp., and twice the ECOFF for *E. coli* (EUCAST, 2014), to select for strains with acquired resistance. The same amounts of vegetable were additionally analysed in the presence of 1 µg/ml cefotaxime plus 400 µg/ml phenylboronic acid (Sigma-Aldrich, Zwijndrecht, The Netherlands) to selectively reduce the growth of AmpC-producers (Coudron, 2005; Crompton et al., 1988; Yagi et al., 2005) and therewith to increase the chance of detection of class-A ESBL-producers. After enrichment, 10 µl was streaked on Brilliance *E. coli*/coliform Selective Agar (Oxoid B.V., Landsmeer, The Netherlands) supplemented with 1 µg/ml cefotaxime and incubated anaerobically for 22 h ± 2 h at 37 °C using GENbox jars (BioMérieux

Table 1
Cultivation type and origin of the investigated vegetables.

Vegetable type	Conventional	Organic
Blanched celery (192)	NL (48), non-NL (48) non-NL = Germany (15), Italy (4), Spain (29)	NL (48), non-NL (48) non-NL = Spain (48)
Bunched carrots (190)	NL (48), non-NL (51) non-NL = Germany (18), Spain (33)	NL (47), non-NL (44) non-NL = Italy (44)
Butterhead lettuce (137)	NL (48), non-NL (0)	NL (48), non-NL (41) non-NL = Belgium (4), France (20), Italy (9), Spain (8)
Chicory (96)	NL (48), non-NL (0)	NL (48), non-NL (0)
Endive (188)	NL (48), non-NL (48) non-NL = Spain (48)	NL (48), non-NL (44) non-NL = Italy (12), Spain (32)
Iceberg lettuce (193)	NL (48), non-NL (48) non-NL = Spain (48)	NL (49), non-NL (48) non-NL = France (4), Spain (44)
Radish (120)	NL (48), non-NL (0)	NL (48), non-NL (24) non-NL = Italy (24)
Spring onion (100)	NL (4), non-NL (48) non-NL = Egypt (20), Germany (20), Morocco (8)	NL (48), non-NL (0)
Total (1216)	NL (340), non-NL (243)	NL (384), non-NL (249)

Note: In between brackets are the numbers of items analysed. NL = The Netherlands, non-NL = foreign countries. Vegetables from foreign countries were selected on the basis of availability in the stores.

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