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Extended-Spectrum-β-Lactamase-Producing *Enterobacteriaceae* Isolated from Vegetables Imported from the Dominican Republic, India, Thailand, and Vietnam

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To examine to what extent fresh vegetables imported into Switzerland represent carriers of extended-spectrum-β-lactamase (ESBL)-producing *Enterobacteriaceae*, 169 samples of different types of fresh vegetables imported into Switzerland from the Dominican Republic, India, Thailand, and Vietnam were analyzed. Overall, 25.4% of the vegetable samples yielded one or more ESBL-producing *Enterobacteriaceae*, 78.3% of which were multidrug resistant. Sixty isolates were obtained: *Escherichia coli*, 26; *Klebsiella pneumoniae*, 26; *Enterobacter cloacae*, 6; *Enterobacter aerogenes*, 1; and *Cronobacter sakazakii*, 1. We found 29 isolates producing CTX-M-15, 8 producing CTX-M-14, 7 producing CTX-M-55, 3 producing CTX-M-65, 1 each producing CTX-M-1, CTX-M-3, CTX-M-27, and CTX-M-63, 5 producing SHV-2, 3 producing SHV-12, and 1 producing SHV-2a. Four of the *E. coli* isolates belonged to epidemiologically important clones: CTX-M-15-producing B2:ST131 (1 isolate), D:ST405 (1 isolate), and D:ST38 (2 isolates). One of the D:ST38 isolates belonged to the extraintestinal enteroaggregative *E. coli* (EAEC) D:ST38 lineage. Two of the *K. pneumoniae* isolates belonged to the epidemic clones sequence type 15 (ST15) and ST147. The occurrence of antibiotic-resistant pathogenic and commensal *Enterobacteriaceae* in imported agricultural foodstuffs constitutes a source of ESBL genes and a concern for food safety.

The production of extended-spectrum β-lactamases (ESBLs) is one of the most important mechanisms of antibacterial resistance in *Enterobacteriaceae*. Most ESBLs can be divided into 4 groups: TEM, SHV, OXA, and CTX-M types (1). Currently, CTX-Ms are the most prevalent type of ESBLs described (2, 3). The last decade has seen a rapid and massive global spread driven primarily by their carriage on resistance plasmids and by the spread of extraintestinal pathogenic *Escherichia coli* clones (4, 5). Important clonal lineages include *E. coli* strains belonging to multilocus sequence type 131 (ST131) (often associated with CTX-M-15) and enteroaggregative *E. coli* (EAEC) ST38 (6). In addition to these widespread ESBLs, less frequently occurring ESBLs have been detected on regional scales, e.g., GES, PER, and VEB types (7).

In recent years, it has been widely recognized that the dissemination of ESBL-producing bacteria is an issue that is no longer restricted to the medical/health care system but represents a growing problem involving food safety and environmental integrity. There is increasing evidence that antimicrobial drug use in the livestock sector plays an important role in the contamination of food with ESBL-producing bacteria (8, 9), but little is yet known about the burden of ESBL-producing *Enterobacteriaceae* on fresh vegetables. In the crop production sector, products can be contaminated through application of manure (animal origin) or sewage sludge (human origin) to the soil or through application of treated or untreated wastewater that is used for irrigation of crops (10).

In Switzerland, as in most industrialized countries, preharvest intervals (i.e., intervals between application of manure to the soil and the subsequent growth phase) restrict the application of manure to the soil, and wastewater is treated before reuse, with high ecological standards and levels of hygiene applied at all stages of culture and harvesting (11). Hence, the bacteriological burden of vegetable crops is low. In contrast, in many developing countries, most prominently Vietnam, China, and India, wastewater without treatment or with insufficient treatment is commonly used for agriculture, producing negative effects on human health and the environment (12, 13).

Analyses of alimentary consumption trends in Switzerland record an increase in Asian and Latin American cuisine and point to a demand for fresh produce (14). Import trade statistics show that imports to Switzerland of edible vegetables from India have doubled over the last decade, and those from the Socialist Republic of Vietnam have quadrupled. Over the last 4 years, Switzerland imported an average of 701.25 metric tons per annum of edible vegetables from the Dominican Republic, India, Thailand, and Vietnam (Swiss Federal Customs Administration [FCA] [https://www.swiss-impex.admin.ch/pages/bereiche/waren/query.xhtml]).

The aim of this study was to evaluate the presence of ESBL-producing *Enterobacteriaceae* in vegetables imported from these countries and to characterize isolated strains by (i) antibiotic susceptibility testing, (ii) identification of the *bla* genes, (iii) multilocus sequence typing (MLST) of the *E. coli* and *Klebsiella pneu-*

Received 26 January 2015 **Accepted** 19 February 2015
Accepted manuscript posted online 27 February 2015
Citation Zurfluh K, Nüesch-Inderbinen M, Morach M, Zihler Berner A, Hächler H, Stephan R. 2015. Extended-spectrum-β-lactamase-producing *Enterobacteriaceae* isolated from vegetables imported from the Dominican Republic, India, Thailand, and Vietnam. Appl Environ Microbiol 81:3115–3120. doi:10.1128/AEM.00258-15.
Editor: J. Björkroth
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doi:10.1128/AEM.00258-15

moniae isolates, and (iv) identifying phylogenetic groups of *E. coli* isolates.

MATERIALS AND METHODS

Bacterial sampling. In July and August 2014, 68 samples of raw vegetables imported via the national airport of Zürich were collected by the food control authority of the Canton Aarau, Switzerland. The vegetables consisted of cucumbers, beans, breadfruit, celery leaves, cha-om (climbing wattle; acacia), chilies, curry leaves, dill, eggplants, garlic chives, lemongrass, onions, peppermint leaves, pak-choy (Chinese cabbage), ponnangani (Asiatic pennywort), several types of squash, water mimosa, and water spinach. The countries of origin were the Dominican Republic (49 samples), India (3 samples), and Thailand (16 samples).

In addition, 101 different fresh vegetable types were purchased in the city of Zürich from 7 retail shops specializing in Asian and South American food and from 3 supermarket chains. The vegetables included basil leaves, beans, celery, Ceylon spinach, chilies, coriander, cucumbers, curry leaves, eggplant, lemon grass, moringa pods (fruits of the horseradish tree), okra (marrow), onions, shallots, dill, soy sprouts, and several types of squash. The samples had been imported from the Dominican Republic (1 sample), India (36 samples), Thailand (44 samples), and the Socialist Republic of Vietnam (20 samples).

In total, 169 vegetable samples were collected for analysis: 50 from the Dominican Republic, 39 from India, 60 from Thailand, and 20 from Vietnam.

Microbiological analysis. Of each unwashed vegetable sample, 15 to 20 g were placed in a sterile stomacher bag. The samples were homogenized using a stomacher sample blender and incubated at a 1:10 ratio in *Enterobacteriaceae* enrichment (EE) broth (BD, Franklin Lakes, NJ, USA) at 37°C overnight. For the detection of ESBL producers, chromogenic Brilliance ESBL agar plates (Oxoid, Hampshire, United Kingdom) were inoculated with one loopful of each of the enrichment cultures. The plates were incubated at 37°C for 24 h under aerobic conditions. Colonies with different chromaticities and morphologies were picked from the selective plates and subcultured on sheep blood agar (Difco Columbia blood agar base EH [Becton Dickinson AG, Allschwil, Switzerland], 5% sheep blood [SB055; Oxoid AG, Pratteln, Switzerland]) at 37°C for 24 h. Identification of isolates was either outsourced and achieved by protein profiling using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Mabritec SA, Riehen, Switzerland) for the samples collected from import border control or obtained using the API ID 32 E phenotypic identification system (bioMérieux, Marcy l'Etoile, France) for the samples collected from retail stores. In cases of doubtful results, identification was verified by *rpoB* sequence analysis (15). The identity of *Cronobacter sakazakii* was confirmed by *rpoB*-based PCR as described previously (16). To investigate the putative enteroaggregative properties of *E. coli* ST38, isolates were tested by PCR for the presence of the EAEC transport regulator gene (*aggR*), using primers and conditions described previously (17).

ESBL confirmation and antimicrobial susceptibility testing. ESBL production was confirmed using Etest-ESBL strips containing cefotaxime, ceftazidime, and cefepime, alone and in combination with clavulanic acid (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Additionally, the presence of β-lactamase was verified with the colorimetric β Lacta test kit (Bio-Rad, Cressier, Switzerland), as described previously (18) and according to the manufacturer's instructions.

Isolates were subjected to susceptibility testing against 13 antimicrobial agents by the disc diffusion method according to CLSI protocols and evaluated according to CLSI criteria (19). The panel included ampicillin (AM), amoxicillin-clavulanic acid (AMC), cephalothin (CF), cefotaxime (CTX), nalidixic acid (NA), ciprofloxacin (CIP), gentamicin (GM), kanamycin (K), streptomycin (S), sulfamethoxazole (SMZ), trimethoprim (TMP) tetracycline (TE), and chloramphenicol (C) (Becton, Dickinson, Heidelberg, Germany). Strains exhibiting resistance to three or more classes of antibiotics were defined as multidrug resistant (MDR).

Molecular biological analysis of β-lactamase genes. Isolates identified as potential ESBL producers were further analyzed by PCR. DNA was extracted by a standard heat lysis protocol and analyzed by PCR for the presence of *bla* genes. Synthesis of primers and DNA custom sequencing were carried out by Microsynth (Balgach, Switzerland). Purification of amplicons was performed using a PCR purification kit (Qiagen, Courtaboeuf, France). Screening for bla_{TEM} and bla_{SHV} was carried out using primers described previously (20), and the resulting amplicons were custom sequenced. Screening for bla_{CTX-M} alleles belonging to CTX-M groups 1, 2, 8, 9, and 25 was done as described by Woodford et al. (21). Amplicons for sequencing individual open reading frames belonging to groups 1, 2, and 9 were generated using primers described previously (8). Group 8 bla_{CTX-M} genes were amplified using the newly designed primers gr. 8 CTX-M-fw (5′-ATG AGA CAT CGC GTT AAG CGG ATG-3′) and gr. 8 CTX-M-rev (5′-CAC GAC GAC TTT CTG CCT TCT GC-3′). The *C. sakazakii* isolate was additionally tested by PCR for the presence of bla_{VEB} (22).

Nucleotide sequences were analyzed with CLC Main Workbench 7.0.2. Database searches were performed using the BLASTN program of NCBI (http://www.ncbi.nlm.nih.gov/blast/).

Phylogenetic classification of *E. coli* isolates. DNAs from *E. coli* isolates were subjected to triplex PCR targeting the *chuA* gene, the *yjaA* gene, and an unspecified DNA fragment termed TspE4.C2, as described previously (23). Each isolate was assigned to one of the four phylogenetic groups designated A, B1, B2, and D. Groups A and B1 typically contain commensal *E. coli* strains, while groups B2 and D consist of virulent extraintestinal strains (24).

Multilocus sequence typing of *E. coli* and *K. pneumoniae*. Multilocus sequence types of *E. coli* isolates were determined as described by Wirth et al. (25). Sequences were imported into the *E. coli* MLST database website (http://mlst.ucc.ie/mlst/dbs/Ecoli) to determine multilocus sequence types.

MLST of the *K. pneumoniae* isolates was performed according to previously described methods (26). Sequence types were determined according to the MLST database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

Alleles and STs that had not been previously described were submitted to the curators of the databases and were assigned new designations.

RESULTS

Prevalence of ESBL-producing *Enterobacteriaceae* in imported vegetables. Overall, 43 (25.4%) of the 169 vegetable samples yielded ESBL-producing *Enterobacteriaceae*. They included 11 (22%) of the 50 samples collected from the Dominican Republic, 13 (33.3%) of the 39 samples from India, 11 (18.3%) of the 60 samples from Thailand, and 8 (40%) of the 20 samples from Vietnam. ESBL producers were detected in 25% of the samples collected at the airport and in 25.7% of the retail store samples. Of the 43 contaminated vegetables, 14 (32.6%) contained multiple isolates (two or more distinct ESBL producers). The types of contaminated vegetables, their origins, and the number of isolates per sample are shown in Fig. 1.

In total, 60 ESBL producers were retrieved. Of these, 26 (43.3%) were identified as *E. coli*, 26 (43.3%) were classified as *Klebsiella pneumoniae* subsp. *pneumoniae*, 6 (10%) were *Enterobacter cloacae*, 1 (1.7%) was *Enterobacter aerogenes*, and 1 (1.7%) was *C. sakazakii* (Fig. 1).

ESBL genes. All 60 isolates were characterized with respect to their ESBL genotypes. Overall, bla_{CTX-M} genes were detected in 51 (85%) strains. Thirty-eight of the bla_{CTX-M} genes belonged to CTX-M group 1 (74.5% of the bla_{CTX-M} genes) and 12 (23.5%)

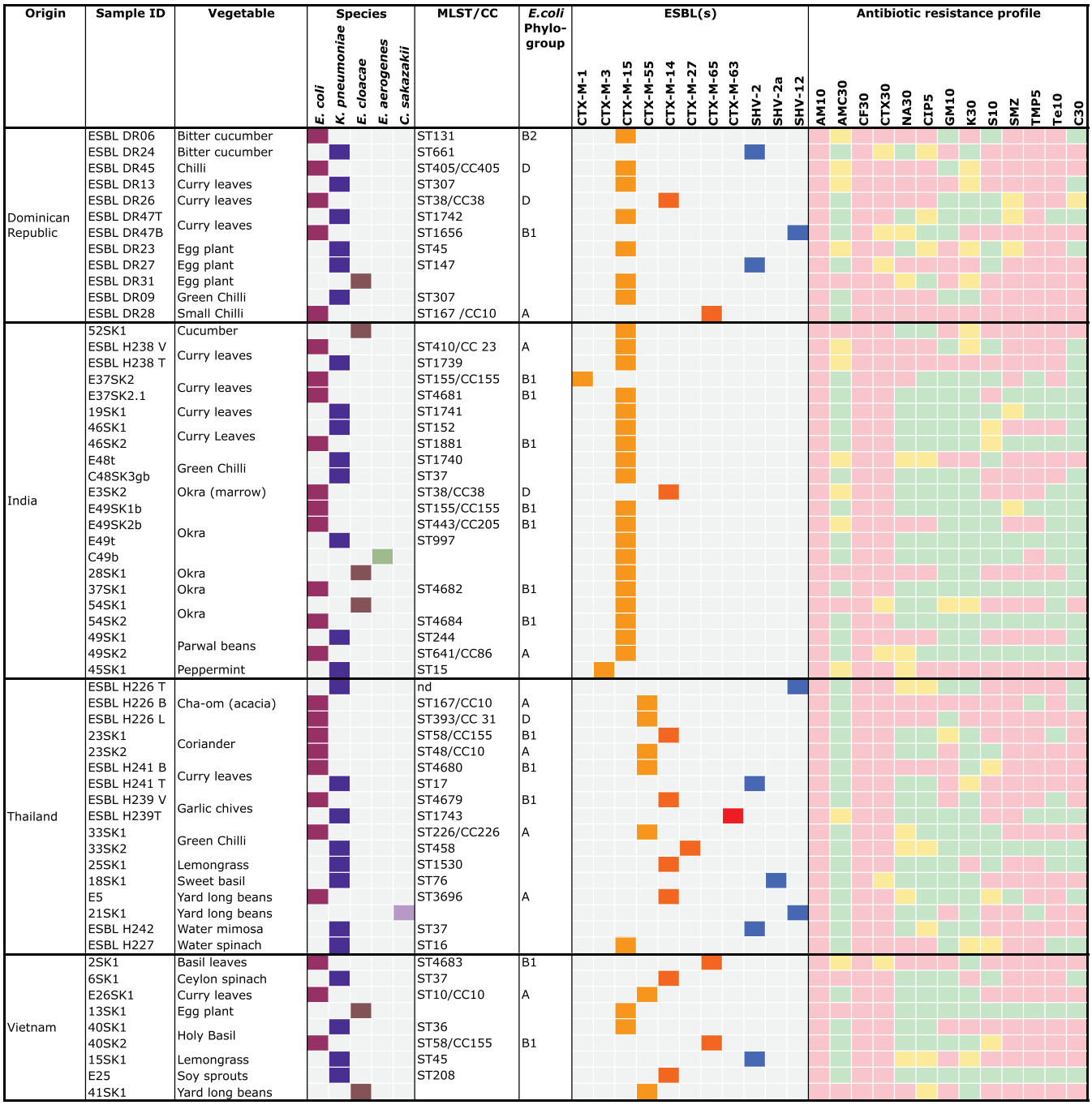

FIG 1 Source data, identities, and distributions of sequence types, clonal complexes, *bla* genes, and antibiotic susceptibility patterns of ESBL-producing *Enterobacteriaceae* isolated from fresh vegetables imported from the Dominican Republic, India, Thailand, and Vietnam. The colors of the squares categorizing ESBLs are as follows: light orange, CTX-M group 1; dark orange, CTX-M group 9; red, CTX-M group 8; blue, SHV enzymes. The colors of the squares categorizing antibiotic resistance profiles are as follows: pink, resistant; yellow, intermediate; green, susceptible. MLST, multilocus sequence type; CC, clonal complex; ID, identifier. The numbers after the drug abbreviations under "Antibiotic resistance profile" represent the amount of drug (in μg) on the discs.

belonged to CTX-M group 9. One representative of CTX-M group 8 was detected (2%). No genes from CTX-M group 2 were found.

Nine strains were identified as SHV-type ESBL producers. Five (55.6%) were SHV-2, and three (33.3%) were SHV-12. One (11.1%) SHV-2a producer was detected.

Overall, of the 26 *E. coli* isolates, 17 (65.8%) *E. coli* strains produced CTX-M group 1 ESBLs and 8 (30.8%) produced CTX-M group 9 ESBLs. Ten (38.5%) harbored $bla_{\text{CTX-M-15}}$, six (23%) $bla_{\text{CTX-M-55}}$, five (19.2%) $bla_{\text{CTX-M-14}}$, and three (11.5%) $bla_{\text{CTX-M-65}}$. One isolate (3.8%) tested positive for $bla_{\text{CTX-M-1}}$, and one (3.8%) harbored SHV-12.

Of the 26 *K. pneumoniae* isolates, 14 (53.8%) *K. pneumoniae* strains produced CTX-M group 1 ESBLs and 5 produced (19.2%) CTX-M group 9 ESBLs. One isolate (3.8%) produced a CTX-M

group 8 ESBL, 13 (50%) harbored $bla_{CTX\text{-}M\text{-}15}$, and 3 (11.5%) carried $bla_{CTX\text{-}M\text{-}14}$. One isolate (3.8%) harbored $bla_{CTX\text{-}M\text{-}3}$, one $bla_{CTX\text{-}M\text{-}27}$, and one $bla_{CTX\text{-}M\text{-}63}$.

Five *K. pneumoniae* (19.2%) isolates harbored SHV-2, one (3.8%) carried SHV-2a, and one carried SHV-12. As an incidental finding, it was noted that the non-ESBL genes $bla_{SHV\text{-}26}$ and $bla_{SHV\text{-}36}$ and bla_{LEN}-like and $bla_{OKP\text{-}5a}$-like genes were present in several *K. pneumoniae* isolates (data not shown).

The *E. cloacae* isolates harbored the CTX-M group 1 gene $bla_{CTX\text{-}M\text{-}15}$ in five cases (83.3%). One isolate carried $bla_{CTX\text{-}M\text{-}55}$.

The *E. aerogenes* isolate harbored $bla_{CTX\text{-}M\text{-}15}$, and the *C. sakazakii* isolate carried $bla_{SHV\text{-}2}$.

Regarding the geographical distribution of the ESBLs, CTX-M group 1 enzymes were detected in 7 of 12 isolates (58.3%) from the Dominican Republic and in 19 of 22 isolates (86.3%) from India. In both countries, CTX-M-15 was the predominant enzyme. In contrast, CTX-M group 9 enzymes were detected more frequently from isolates from Thailand and from Vietnam (5 of 17 isolates [29.4%] and 4 of 9 isolates [44.4%], respectively).

Notably, none of the isolates originating from India contained any SHV ESBLs.

Antimicrobial susceptibility patterns. Disc diffusion tests showed that all 60 isolates were resistant to ampicillin and to the narrow-spectrum cephalosporin cephalothin. Resistance to cefotaxime was noted for 53 (88.3%) of the isolates.

Disc diffusion tests performed for other categories of antibiotics revealed that 19 (31.7%) isolates were resistant to the quinolone antibiotic nalidixic acid and 18 (30%) were resistant to the fluoroquinolone ciprofloxacin. Resistance to aminoglycosides was detected in 20 (33.3%) isolates resistant to gentamicin, 12 (20%) resistant to kanamycin, and 31(51.7%) resistant to streptomycin. Resistance to the folate pathway inhibitors sulfamethoxazole and trimethoprim was noted in 44 (73.3%) isolates and 45 (75%) isolates, respectively. Tetracycline resistance was found in 39 (65%) and chloramphenicol resistance in 28 (46.7%) isolates, respectively.

Multidrug resistance was detected in 47 (78.3%) of the isolates: 11 isolates (91.6%) from the Dominican Republic, 13 (59%) of the 22 isolates from India, 16 (94%) of the 17 isolates originating from Thailand, and 7 (77.8%) of the 9 strains from Vietnam.

Epidemiological characteristics of *E. coli* and *K. pneumoniae* isolates. (i) Phylogenetic groups and MLST of *E. coli*. Phylogenetic typing allocated 21 (80.8%) of the *E. coli* isolates to group A or B1, which typically contain commensal *E. coli* strains. Five isolates (19.2%) belonged to extraintestinal pathogenic phylogroups B2 and D (one and four isolates, respectively).

Multilocus sequence typing of the 26 *E. coli* isolates identified 22 different sequence types (Fig. 1). There were four new allelic combinations (isolates E37SK2.1, 37SK1, ESBL H241 B, and ESBL H239 V). Two isolates contained new allelic variants of the *fumC* and *recA* genes: isolate 54SK2 with ST4684 (*fumC*, allele number 604) and isolate 2SK1 ST4683 (*recA*, allele number 326).

Among the pathogenic groups B2 and D, four isolates belonged to the epidemiologically important sequence types ST131, ST405, and ST38: isolate ESBL DR06 was assigned to the internationally disseminated CTX-M-15-producing B2:ST131 clone; isolate ESBL DR45 belonged to D:ST405, which belongs to the clonal complex CC405; and two isolates, ESBL DR26 and E3SK2, were identified as D:ST38, which belongs to the clonal complex CC38. Since this clone may include enteroaggregative *E. coli* strains, these two isolates were tested by PCR for the EAEC-specific marker gene *aggR*. The results revealed that one isolate (E3SK2) belonged to the extraintestinal EAEC D:ST38 lineage. One further isolate (ESBL H226 L) was classified as D:ST393.

(ii) MLST of *K. pneumoniae*. Multilocus sequence typing revealed high diversity among the 26 *K. pneumoniae* isolates. Five isolates exhibited new sequence types (isolates ESBL H238T, E48T, 19SK1, ESBL DR47T, and ESBL H239T). Two isolates, 45SK1 and ESBL DR27, belonged to epidemic clones ST15 and ST147, respectively. For isolate ESBL H226 T, the ST could not be determined, because the *mdh* gene could not be amplified.

DISCUSSION

Recent studies indicate that fresh vegetables constitute a source of ESBL producers and represent a possible route for the dissemination of resistance genes via the consumer in the community (27–29).

Vegetable crops originating from most European and North American countries are farmed according to regulations for applying manure/slurry to protect vegetables from contamination with pathogenic microorganisms, in accordance with the recommendations of the World Health Organization (WHO) (30). Consequently, carriers of bla_{ESBL} and multidrug resistance genes associated with vegetables have been described as predominantly saprophytic and opportunistic bacteria, which are thought to constitute a background reservoir of antibiotic resistance genes (31) and not a threat *per se* to human health.

In this study, we examine the presence of ESBL-producing *Enterobacteriaceae* in fresh vegetables imported into Switzerland from countries with very different farming standards and where the food production industry is to a certain extent underdeveloped.

The high rate of contamination (average, 25.4%) of the samples with ESBL producers and the very high rate (78.3%) of MDR *Enterobacteriaceae* detected in this study give rise to concern. These results contrast strongly with results from similar studies that reported lower prevalences (6% to 12%), of ESBL producers in raw vegetables (27, 28, 32).

We found national variations among the CTX-M types identified in the samples. The predominance of $bla_{CTX\text{-}M\text{-}15}$ genes in isolates from India is in accordance with previous studies involving clinical isolates originating from Delhi and south India, and the frequency of group 9 CTX-M types in isolates from Thailand and Vietnam is reflective of reports from China and the Far East (3, 33). In the isolates from Thailand analyzed in this study, CTX-M-55 outnumbered CTX-M-14. Originally detected in clinical isolates of *E. coli* and *K. pneumoniae* from Thailand in 2005 (34), this particular ESBL type has been found widely in food-producing animals and humans in China and appears to be displacing CTX-M-14 as the most common CTX-M variant (35). Our data indicate that this epidemiological characteristic may hold true for Thailand and also for Vietnam. In comparison, the CTX-M type distribution of ESBL producers isolated from healthy humans in Switzerland is dominated by CTX-M-15 and CTX-M-1 (36).

The predominance of phylogenetic groups A and B1 among the *E. coli* isolates and the wide diversity of multilocus sequence types among the *E. coli* and *K. pneumoniae* isolates indicate that bla_{ESBL} and MDR genes are well established in commensal strains. It is already recognized that commensal bacteria constitute an important reservoir of antibiotic resistance genes in food animals

(37). Our results suggest that vegetables of the types and origins analyzed in this study represent another potent and hitherto underappreciated source of antibiotic resistance genes.

The occurrence of pathogenic bacteria in food is a threat to public health. In this study, we found a CTX-M-15-producing isolate belonging to the highly virulent pandemic *E. coli* strain B2:ST131, which is associated with severe infections in humans (38). Furthermore, one *E. coli* D:ST405, two *E. coli* D:ST393, and two *E. coli* D:ST38 strains were found in this study. These strains also belong to lineages that cause extraintestinal diseases, mainly urinary tract infections, in humans and contribute to the global dissemination of ESBLs and MDR genes (6, 39). The detection of enteroaggregative properties in one of the *E. coli* D:ST38 strains is of particular concern. EAEC is associated with acute or persistent diarrhea in outbreak and nonoutbreak settings worldwide. Its association with CTX-M-14 has been described recently in Europe (40, 41), as well as in Asia (42), and its detection in vegetables destined for human consumption raises questions concerning food safety.

C. sakazakii is an opportunistic foodborne pathogen that can cause fatal necrotizing enterocolitis, bacteremia, and meningitis in infants and immunocompromised adults (43), and its detection, for the first time to our knowledge, as an SHV-12 producer in a vegetable sample from Thailand merits attention. Previously, a clinical isolate of *C. sakazakii* harboring $bla_{\text{VEB-1}}$, a bla_{ESBL} gene found increasingly in Thailand, was reported (22). However, the isolate in this study tested negative for this particular gene.

Among the *K. pneumoniae* isolates detected in this study, two (45SK1 and ESBL DR27) belonged to epidemic clones associated with nosocomial infections in humans (44, 45), giving rise to further concern for consumer health.

In conclusion, the results of this study suggest that the international production of and trade in fresh vegetables constitute a possible route for the spread of ESBLs and pathogenic *Enterobacteriaceae*. Appropriate measures, such as the improvement of agricultural practices and water quality, need to be taken, and globally mandatory guidelines should be established in order to ensure consumer and public health worldwide.

ACKNOWLEDGMENTS

This work was partly supported by the Swiss Federal Office of Public Health, Division of Communicable Diseases.

We thank the team of curators of the Institut Pasteur MLST (*Klebsiella*) and the MLST database at the University of Warwick (*E. coli*) for importing novel alleles and Beatriz Winkenbach for technical assistance.

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