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Original article

Prevalence of, and Genetic Relationship Between, Coliforms in Food, Foodhandlers and Contact Surfaces

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Summary: Coliforms have been used as indicator organisms in examinations of food for fecal contamination and for the identification of unsanitary conditions in food outlets. In the present study we determine the prevalence of coliforms isolated from foods, kitchen utensils and the hands of those involved in the preparation of food. The study is based on, samples collected from 100 food items, 45 foodhandlers' hands, 27 cutting boards and 40 knife handle surfaces in six different canteens on a university campus during the 2017- 2018 academic year. The presence of coliforms was confirmed with biochemical tests and the PCR amplification of 16s rRNA. Gradient PCR was used subsequently for isolation followed by a sequence analysis to determine any association. In all, 14 *Escherichia coli*, 32 *Klebsiella pneumoniae* and six *Citrobacter* spp. isolates were identified from the foodhandler's hands, foods and surfaces in a gradient PCR and sequence analysis. Furthermore, *E. coli* isolates were found on the hands and, cutting boards, and in Turkish sausage, grilled chicken, sauted chicken, Urfa kebab and, chicken doner; Klebsiella *pneumoniae* was isolated from hands, grilled chicken, knife handles, meatballs and, cutting boards; and *Citrobacter* spp. was obtained from hands, Turkish sausage, cutting boards and, grilled chicken.

Key words: Coliforms, Contamination, *E. coli*, Food, Genotyping

Introductıon

Ready-to-eat foods (RTE) have played a major role in raising awareness of food poisoning outbreaks. RTE foods are consumed by different age groups, including students and teens (1). The processing of RTE foods requires comprehensive handling, and they are usually prone to cross contamination from soil, water, air, storage/distribution resources, the environment and human activities (2). Food prepared in large amounts in particular is at high risk of contamination and may cause food-borne outbreaks unless basic hygiene guidelines are followed (3, 4, 5).

The members of the Enterobacteriaceae family that can be found in RTE food (6) can lead to acute gastroenteritis and may be responsible for most of the disease outbreaks around the world (7, 8). The presence of coliforms can indicate a deficiency in the sanitary practices of those preparing the food (9, 10). Contamination often occurs by the fecal-oral route when pathogens exist in the feces of ill or subclinical persons. Workers can spread pathogens during the early stage of illness or can be long-term asymptomatic carriers (11, 12). Food contact surfaces can act as reservoirs for the bacterial contamination of RTE food and should be washed and disinfected regularly to prevent

bacterial accumulation and spread (13). The present study investigates the prevalence of coliform and fecal coliform bacteria in RTE foods, food handlers and food contact surfaces, and identifies the genetic relationship between these isolates. To this end, the study investigates sources of contamination of foods in university campus canteens through a sequence analysis of isolates, and possible relationships were established.

Materials And Methods

Microbiological sampling of surfaces, cooking utensils and food handlers' hands

Food samples (n=100) weighing 100 g were collected in sterile bags and brought to the laboratory under cold chain, were and analyzed within 1-2 hours. Then, 10g of each food samples was collected aseptically and transferred to sterile plastic pouches and homogenized for 60 s in 90 mL of sterile peptone water (1 g/L). After the serial dilutions of the samples, they were inoculated into growth media using the standard drop-plate method. A total of six canteens across the university campus, employing 45 food handlers (including permanent and contract workers), were examined for the study. Hand samples were collected from the thumb and forefinger through a "pressing the finger into the petri dish" method, for which. RODAC petri dishes containing a Chromocult Coliform Agar (Merck 1.10426) were used. The petri dishes containing the samples were incubated at 37 °C for 24 h. Samples were taken from the food contact surfaces (27 slicing boards, 40 knife handles) using a sterile 15 cm^2 frame, which was used to outline the area where the swabbing was carried out. The swab was then placed in a tube containing 10 mL maximum recovery dilution with 0.05% Sodium thiosulfate (MRD, Oxoid CM733) and stored in an ice container. The samples were analyzed within 2 h. Coliform and *E. coli* counts were made after incubation in Chromocult-Coliform-Agar 37 °C for 24 h. The identification of coliform bacteria was carried out via biochemical tests (14). We did not use a reference strain in this study. Because it is determined which strain the isolates are from the DNA sequence.

PCR and Sequencing Protocol

A gradient PCR was performed in collected aplicons in order to determine the binding temperature (Melting Temperature, Tm) and, a Tm value of 60 degrees was determined. Total genomic DNA was isolated using a commercial spin column kit (Thermo Fisher, K0722). Primers were designed using the Fast PCR Professional 6.1.2 package program, and the dimer and hairpin formation between the primers was controlled using the same program. Isolated DNA was amplified using the forward primer F46 (5´-ACCAAGTCTCAAGAGTGAACACG-3´) and R1585 (5´-TCACAAAGTGGTAAGCGCCCTC-3´), and 16S rRNA genes were used to evaluate the coliform bacteria. In total, 10 µl containing a 1xPCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTP, 0.25 mM forward and reverse primer, 1U Platinum Taq DNA polymerase (Invitrogen, 10966034) and 20 ng DNA were included in the PCR reactions performed in a thermocycler (Applied Biosystems Veriti). The PCR protocol was set to two cycles for 2 min at 94 °C, 45 sec at 94 °C, 30 sec at 60 °C, 1 min at 72 °C and 35 cycles at 72 °C for 10 min, respectively. The amplification products were analyzed by gel electrophoresis in 2% agarose gel followed by ethidium bromide staining and purging with Exonuclease I (Thermo Fisher, EN0581) and FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher, EF, EF0652). The aplicon sequence was performed in accordance with the BigDye Therminator 3.1 kit protocol. The products obtained following PCR amplification were purified with the ethanol / EDTA / sodium acetate precipitation method. Then, 15 µl of Hi-Di formamide was added to each well, and the sample was loaded onto the DNA Sequence Analyzer (ABI 3500). After the DNA sequencing analysis, all sequences were edited (Sequencher 5.4.6) and aligned using the Bioedit Sequence Alignment Editor Analysis Program, and the amplification product was read as bi-directional 1426 bp. We then compared the genotypic results and similarity searches were carried out using MEGA 4. The evolutionary relationships of pathogens based on 16S rRNA were inferred using the neighbor-joining method (15). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the analyzed

taxa. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa were clustered in the bootstrap test (1000 replicates) is shown next to the branches (16). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and a dendrograms was constructed in MEGA4 (17, 18).

Results

All analyses of coliform and *E. coli* were applied using one primer set, and the generated PCR products were 1426 bp. The relationships between the presence of organisms on the hands, surfaces and in food were calculated in order to establish whether cross-contamination was apparent among three surfaces. The relationship between isolates is shown on the dendrogram (Figure). The present study identified a genetic relationship between *E. coli*, *Klebsiella pneumoniae* and the *Citrobacter* spp. isolates obtained from the food, the foodhandlers' hands and the food preparation equipment through genotyping characterization. The isolates were derived from 19 hands (42.2%), 19 foods (19%) and 14 contact surfaces (20.8%). A sequence analysis revealed a correlation among some strains, as well as the source of the isolates. According to these results, *E. coli* and *Citrobacter* spp. were present on food handlers' hands, and the presence of *Klebsiella pneumoniae* in the food and hand samples revealed a 99-100% similarity in a blast comparison in the 2nd canteen. In the third canteen, *Klebsiella pneumoniae* was detected only on the hands, and these isolates were similar to *Klebsiella* strains from the other canteens. A 99-100% similarity was observed in the *Klebsiella pneumoniae, Citrobacter freundii* and *E. coli* isolated from hand and Turkish soujouk samples in the 4th canteen. In the 6th canteen, the isolates (*Klebsiella pneumoniae* and *E. coli*) obtained from the foods, hands and cutting boards were the same. In addition, the *Citrobacter freundii* isolated from grilled chicken was found to be identical to that isolated from the 4th canteen. No isolates were obtained from canteens 1 and 5. Considering all these results, the isolates recovered from 2nd, 3rd, 4th and 6th canteens were 97-100% similar and the identified (Table 1). Pathogens namely Enterobacteriaceae such as *E. coli* and other coliforms, as well as members of the genera *Proteus* and *Klebsiella* were most associated with poor hygiene practices. Figure 1 shows the phylogenetic tree for pathogen strains based on 16S

Table 1. Closest known isolates related to genetic similarity of 52 food, foodhandler's and food contact surface isolates

Sample	Source	Closest 16S NCBI isolate and accession	Sequence similarity to isolate (%)	Canteen
1	Hand	*Klebsiella pneumoniae* F93-1 (CP026160.1)	100	Canteen 2
2	Hand	*Klebsiella pneumoniae* F93-2 (CP026157.1)	100	
3	Hand	*Klebsiella pneumoniae* CCRI-21711 (CP035540.1)	99	
4	Hand	*Escherichia coli* NCTC11104 (LR134214.1)	99	
5	Hand	*Citrobacter* sp. FDAARGOS_155 (CP014030.2)	99	
6	Grilled chicken	*Klebsiella pneumoniae* F93-1 (CP026160.1)	100	
7	Grilled chicken	*Klebsiella pneumoniae* F93-2 (CP026157.1)	100	
8	Grilled chicken	*Klebsiella pneumoniae* CCRI-21711 (CP035540.1)	99	
9	Hand	*Klebsiella pneumoniae* F93-1 (CP026160.1)	100	Canteen 3
10	Hand	*Klebsiella pneumoniae* F93-2 (CP026157.1)	100	
11	Hand	*Klebsiella pneumoniae* CCRI-21711 (CP035540.1)	99	

Table 1 (*Continued*)

Table 1. Closest known isolates related to genetic similarity of 52 food, foodhandler's and food contact surface isolates (*Continued*)

Sample	Source	Closest 16S NCBI isolate and accession	Sequence similarity to isolate (%)	Canteen
12	Hand	*Klebsiella pneumoniae* F93-1 (CP026160.1)	100	Canteen 4
13	Hand	*Klebsiella pneumoniae* F93-2 (CP026157.1)	100	
14	Hand	*Klebsiella pneumoniae* CCRI-21711 (CP035540.1)	99	
15	Hand	*Escherichia coli* NCTC11104 (LR134214.1)	100	
16	Hand	*Citrobacter freundii* 705SK3 (CP022151.1)	99	
17	Soujouk	*Escherichia coli* U12A (CP035476.1)	100	
18	Soujouk	*Citrobacter freundii* MFC-pH7 (KY434109.1)	99	
19	Hand	*Klebsiella pneumoniae* F93-1 (CP026160.1)	100	Canteen 6
20	Hand	*Klebsiella pneumoniae* F93-2 (CP026157.1)	100	
21	Hand	*Klebsiella pneumoniae* CCRI-21711 (CP035540.1)	99	
22	Knife handle	*Klebsiella pneumoniae* 30660/NJST258_1 (CP006923.1)	100	
23	Knife handle	*Klebsiella pneumoniae* F89-1 (CP026159.1)	99	
24	Knife handle	*Klebsiella pneumoniae* JM45 (CP006656.1)	100	
25	Hand	*Klebsiella pneumoniae* F93-1 (CP026160.1)	100	
26	Hand	*Klebsiella pneumoniae* F93-2 (CP026157.1)	100	
27	Hand	*Klebsiella pneumoniae* CCRI-21711 (CP035540.1)	99	
28	Knife handle	*Klebsiella pneumoniae* F93-1 (CP026160.1)	100	
29	Knife handle	*Klebsiella pneumoniae* F93-2 (CP026157.1)	100	
30	Knife handle	*Klebsiella pneumoniae* CCRI-21711 (CP035540.1)	99	
31	Meatball	*Klebsiella pneumoniae* F93-1 (CP026160.1)	100	
32	Meatball	*Klebsiella pneumoniae* F93-2 (CP026157.1)	100	
33	Meatball	*Klebsiella pneumoniae* CCRI-21711 (CP035540.1)	99	
34	Cutting board	*Escherichia coli* isolate EC-12536 (LR025099.1)	100	
35	Cutting board	*Klebsiella pneumoniae* F81 (CP026164.1)	99	
36	Cutting board	*Klebsiella pneumoniae* NFYY0065 (CP035531.1)	100	
37	Cutting board	*Citrobacter braakii* FDAARGOS_290 (CP022049.2)	97	
38	Cutting board	*Citrobacter youngae* F_46 (MG428756.1)	97	
39	Grilled chicken	*Citrobacter freundii* 705SK3 (CP022151.1)	99	
40	Grilled chicken	*Escherichia coli* NCTC11104 (LR134214.1)	100	
41	Chicken saute	*Escherichia coli* U12A (CP035476.1)	100	
42	Chicken saute	*Escherichia coli* isolate EC-TO75 (LS998785.1)	99	
43	Grilled chicken	*Escherichia coli* U12A (CP035476.1)	100	
44	Grilled chicken	*Escherichia coli* NCTC9702 (LR134246.1)	99	
45	Urfa Kebab	*Escherichia coli* U12A (CP035476.1)	100	
46	Urfa Kebab	*Escherichia coli* NCTC9054 (LR134225.1)	99	
47	Urfa Kebap	*Escherichia coli* NCTC8623 (LR134234.1)	99	
48	Chicken doner	*Escherichia coli* U12A (CP035476.1)	100	
49	Chicken doner	*Escherichia coli* O26 RM10386 (CP028126.1)	99	
50	Cutting board	*Klebsiella pneumoniae* strain FDAARGOS (CP033756.1)	100	
51	Cutting board	*Klebsiella pneumoniae* strain SCKP040074 (CP029388.1)	99.9	
52	Cutting board	*Klebsiella pneumoniae* strain L5-2 (CP025684.1)	99.9	

rRNA. In this tree, *Klebsiella pneumoniae* strain were grouped into two clusters, while the second cluster contained *E. coli* strains and the third cluster contained *Citrobacter* subspecies strains. An *E. coli* EC-12536 strain has been documented in the *Klebsiella* strain and a *Citrobacter freundii* MFC-pH7 strain, which is closely related with *E. coli* strains, has also been identified (Figure 1). The findings ot the study identifying similar strains among the canteens suggests that the same staff rotate among the six canteens and carry these bacteria through careless and improper hygiene practices, as well as fecal contamination. We noticed that the responsible managers of 1st and 5th canteens attach more importance to hygiene and pay more attention to hygiene rules in the toilet and kitchen. In addition, as the 1st and 5th capacity are smaller than other canteens, the food and beverage cycle and product variety were also less.

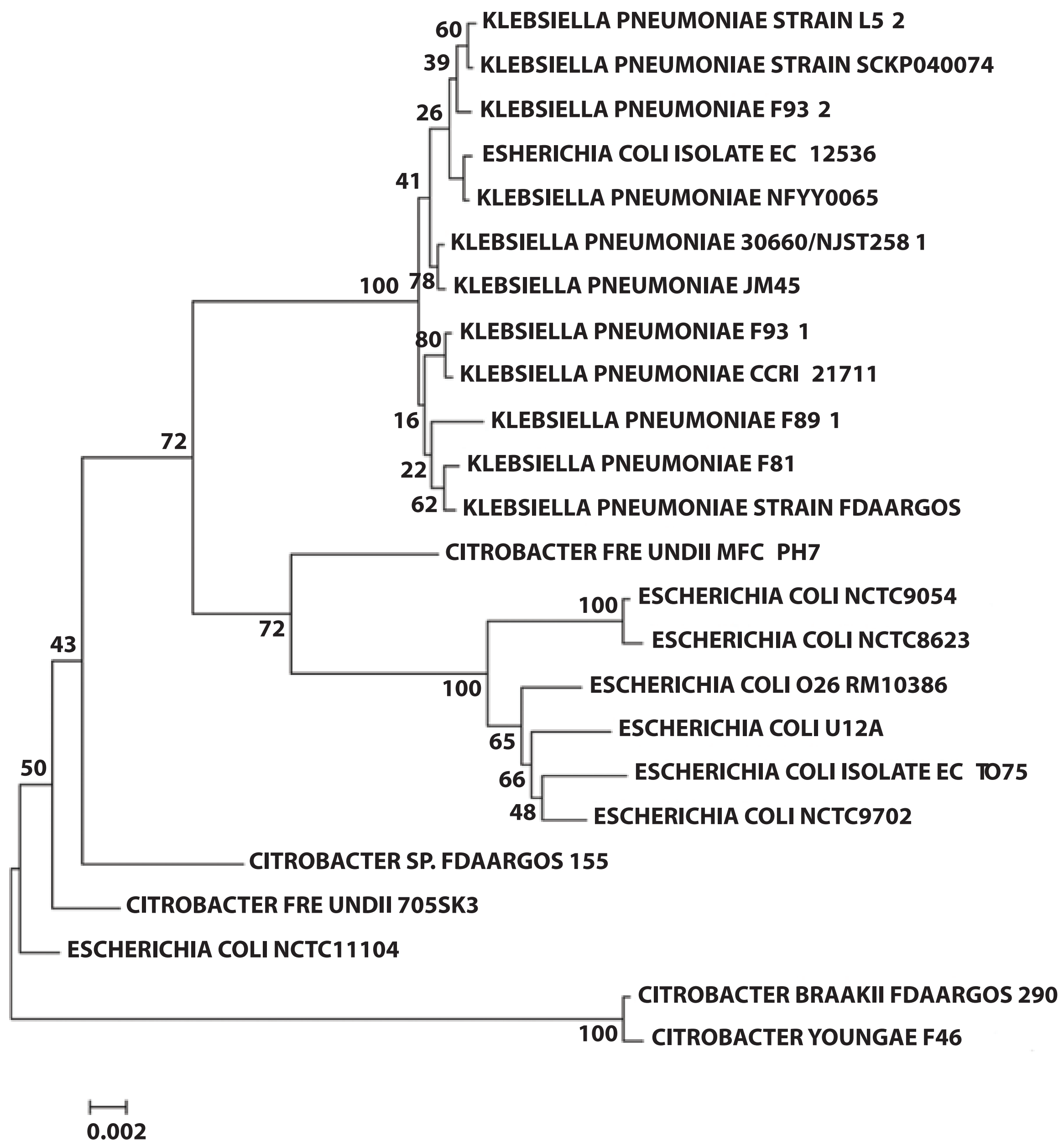

Figure 1. The relationship of isolates on the dendrogram

Discussion

Food-borne bacteria are often found in kitchens lacking sufficient infrastructure and equipment and constitute an important source of foodborne illness (2). A food handler is a person who works in the food business that processes food either directly or indirectly (6, 12). Food-borne diseases are usually caused by the consumption of contaminated or poorly prepared foods and are a serious public health problem worldwide (19, 20, 21). The increased incidence of foodborne diseases has led to a reemergence of interest and attention in the hygiene and cleanliness of public places, canteens, and dining halls (19, 22, 23). Many foodborne disease cases are reported every year around the world, with many factors contributing to high incidents rates (24, 25, 26). Food workers play an important role in public health, especially if the food is not hygienically prepared, preserved or served (4, 27). There are various means by which pathogens are able to survive and multiply in food, such as improper food and sanitation practices and environmental factors (28, 29). Although studies have generally investigated the prevalence of pathogens, there has been no study to date determining the genetic relationship between isolates obtained from food, hands and kitchen equipment. In the present study, we detected that the coliforms and *E. coli* isolates were 97-100% similar in a blast comparison, which indicates that the contamination of foods, hands and surfaces with *E. coli* can be linked to fecal contamination. It is likely that the various populations of employees in diverse locations at different times of the year may have variable hand carriage rates for these pathogens and contaminate surfaces or foods more often (12). In the present study, the food handlers who come into contact with the food were seasonal, part-time, rotating staff and some of them were citizens of other countries. Pamuk et al. (30) reported rates of coliform and *E. coli* isolated from knife handle of 52.5% and 12.5%, respectively. The hand sample results revealed a coliform contamination level of 4.4% ≥2.5 cfu cm^{-2} and 14.7% ≥1.0 cfu cm^{-2} of *E. coli*. When these values are compared those reported in literature, 32% of the food workers exceeded the target value of <2.5 cfu cm^{-2} established in literature (31). Lee et al. (32) revealed that the food handlers approximately 35% (n = 30) had exceeded the standard coliform count (≥10 CFU/cm^2). Ayuningtya et al. (33) reported that snacks with *E. coli* contamination were found at 9.5% (n=7) and many snacks were contaminated with coliform and *E. coli* used chicken in their presentation. Gil et al. (34) found that samples taken from meals, kitchen utensils and, kitchen cloths were contaminated with *E. coli* at a rate of 4%, 16%, and 42%, respectively. It should be noted that dishwashing is an important source of bacterial contamination in food preparation places facilities. High bacterial concentrations (up to 4 log cfu/cm^2 *E. coli*) have been determined in used dishcloths (35), which have been shown to carry the largest load of total coliforms and fecal coliforms (2). These findings indicate that hygiene practices related to kitchenware and hand cleanliness should be improved to reduce contamination. The results show the poor state of food processing among those engaged in the preparation of food and highlight the importance keeping surfaces clean to prevent the potential transfer of pathogens to food. There is very little data on the genetic association of pathogenic bacteria in hands, surface, and foods. According to the information garnered through this research, taking the samples into consideration, it can be said that the identification of the same isolates from different canteens means that it is likely that the pathogens are being transmitted from the hands to the food and to the kitchen equipment.

Conclusion

To prevent foodborne illnesses, it is necessary to store foods under suitable conditions, to have hygienic food preparation facilities and to employ appropriate personnel that have knowledge of hygiene. Furthermore, food processor and food vendor training programs at the point of sale of food are very important. This study indicates that there is a high probability of contamination from pathogens passing from the hands to various surfaces, which underlines the need for effective hand hygiene. Thorough and continuous handwashing, sterilization of equipment, care for the environment and the use of appropriate packaging materials can prevent the spread of bacteria which are

important parameters for food safety. In addition, the adoption and implementation of the HACCP (Hazard Analysis Critical Control Point) principle should be considered a priority.

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