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Occurrence and characterization of *Escherichia coli* in raw lettuce consumed in a military hospital

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Abstract - A healthy diet requires raw vegetable intake. However, *Escherichia coli*, one of the bacteria constituting the human and animal intestinal microflora, sometimes contaminate vegetables, including lettuce. In places where food is prepared on a large scale, the presence of *Escherichia coli* constitutes a reliable indication of fecal contamination. The aim of this study was to assess the levels of *Escherichia coli* contaminating 100 raw lettuce heads destined for consumption at a military hospital, and to characterize the 27 isolates recovered therefrom, with regard to their susceptibility to antibiotics as well as their phylogenetic groups and the occurrence of class 1 and 2 integrons. Ten specimens were bacteriologically non-satisfactory. Furthermore, 17 isolates (63%) were sensitive to all antibiotics tested, whereas 10 isolates (37%) were resistant to different antibiotics. Among these, 18.5% were resistant to 3 antibiotics, 11.11% were resistant to 3 other antibiotics and 7.4% were resistant to yet 2 other antibiotics. The majority of isolates (74%) segregated into phylogenetic groups A and B1, suggesting that they are commensal strains. However, 26% fell into groups B2 and D, hence constituting potentially virulent entities. Among the isolates, 51.8% harboured class 1 integrons, while 48.15% were devoid of integrons. The isolates exhibiting resistance to different antibiotics carried class 1 integrons. Our study revealed high levels of *E.coli* contaminating lettuce, thus a potential risk for foodborne outbreaks. The isolates displayed resistance to different antibiotics correlating with the presence of class 1 integrons, and are armed with a pathogenic potential due to strains of B2 and D groups.

Key words: Lettuce, Antibiotic resistance, *Escherichia coli*, phylogenetic group, integrons.

1. Introduction

Intake of raw vegetables is important for a healthy diet as they provide vitamins, antioxidants and minerals. However, these advantages are sometimes counteracted by their ability to cause foodborne illnesses when contamined by harmful bacteria. Vegetables can be contaminated during their production, *i.e.* culture, or during their processing, from harvest to the consumer's table. This sometimes occurs through a simple contact during their manipulation, an event known as cross-contamination. Contaminated raw vegetables also provide a favorable environment for bacterial growth, thus constituting an important source for human infection.

Escherichia coli (*E. coli*), a Gram-negative bacteria belonging to the *Enterobacteriaceae* family, is among the micro-organisms that contaminate raw vegetables. *E. coli* is a commensal bacteria of the intestinal tract. However, depending on the strain and the host, it can generate severe intestinal and extra-intestinal diseases. Intestinal symptoms appear 3-5 days after ingestion of contaminated food. They can be benign, similar to those of a simple flu, but can also manifest as severe abdominal cramps accompanied by vomiting and fever, while in more severe cases the infection can lead to kidney failure. Serious complications, like in other infectious diseases, affect mostly vulnerable individuals, including infants, the elderly and the immunocompromised.

It is however, practically difficult to prevent all *E. coli* contaminations, even under observance of hygienic rules. Therefore, antibiotics, including β-lactam-based compounds, quinolones and

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aminosides are considered as optimal solutions for treating intestinal and extra-intestinal infections. However, this decade has been marked by the emergence and dissemination of *E. coli* strains that are resistant to antibiotics, thus compromising treatment. Antibiotic resistance, a serious threat to public health safety, is carried by mobile genetic elements, such as integrons and plasmids.

Development of more sophisticated bacteriological technologies, combined with the use of molecular biology in clinical microbiology laboratories, enable a clear improvement in identification of pathogens, the number of which is alarmingly increasing, and provide crucial information useful for control of infections.

In this context, our present studies aimed at determining the extent of *E. coli* contamination of raw vegetables (lettuce heads) consumed in conjunction with large-scale food preparation in the Military Hospital of Tunis, as well as providing a phenotypic and a genotypic characterization of the bacteria. Our investigations enabed us to assess antibiotic susceptibility of a collection of 27 *E. coli* isolates from contaminated raw lettuce heads, identify their phylogenetic groups and determine the ocurrence of class 1 and 2 integrons.

2. Materials and Methods

2.1 Sampling

Samples were recovered from 100 fresh lettuce heads in the kitchen of the Military Hospital in Tunis between April and July in 2013. Samples were taken out aseptically by a professional in food hygiene and kept cool during transfer to our laboratory. Upon arrival, undamaged fresh specimens were recorded before being processed. The samples were prepared according to the ISO 16649-1: 2001 standard.

2.2 Antibiotic susceptibility testings

Generation of an antibiogram enables *in vitro* determination of bacterial sensitivity to antibiotics. We generated antibiograms by using the agar diffusion method (Mueller-Hinton agar; Biokar Diagnostics) according to recommendations of Le Comité d'Antibiogramme de la Société Française de Microbiologie (CA-SFM, 2013).

Table 1. Distribution of *E. coli* phylogenetic groups and subgroups based on the presence (+) or absence (-) of specific DNA bands after gel electrophoresis. Adapted from Clermont et al. (2000) and Escobar-Paramo et al. (2006).

Chu A (279bp)	*Yja A* (211bp)	*Tsp E4C2* (152bp)	Phylogenetic groups	Subgroups
–	–	–	A	A_0
–	+	–	A	A_1
+	+	–	B2	$B2_2$
+	+	+	B2	$B2_3$
+	–	–	D	D_1
+	–	+	D	D_2
–	(±)	+	B1	B1

2.3 Molecular characterization of *E. coli* isolates

We used the Polymerase Chain Reaction (PCR) assay to determine the phylogenetic groups and their distribution as well as the presence or absence of class 1 and 2 integrons in our lettuce-derived isolates.

2.3.1 Determination of the phylogenetic groups by PCR

E. coli strains segregate into four main phylogenetic groups (A, B1, B2, and D). Categorization of *E. coli* strains into a specific group is efficiently done by PCR which enables visualization of the presence or absence in their genome of three molecular markers, including the *chuA* and the *yjaA* genes as well as the DNA fragment TspE4C2 (Table 1), as demonstrated in previous studies (Clermont et al., 2000; Escobar-Paramo et al., 2006). The primers used to detect these genes (Clermont et al., 2000) are shown in Table 2.

Table 2. Primer sets and amplified fragments for identification of *E. coli* phylogenetic groups

Locus	Primers	Size
ChuA	*ChuA.1*: 5'-GAC GAA CCA ACG GTC AGG AT-3' *ChuA.2*: 5'-TGC CGC CAG TAC CAA AGA CA-3'	279 bp
YjaA	*YjaA.1* : 5'-TGA AGT GTC AGG AGA CGC TG-3' *YjaA.2* : 5'-ATG GAG AAT GCG TTC CTC AAC-3'	211 bp
TspE4C2	*TspE4C2.1*: 5'-GAG TAA TGT CGG GGC ATT CA-3' *TspE4C2.2*: 5'-CGC GCC AAC AAA GTA TTA CG-3'	152 bp

2.3.2 Detection and characterization of class 1 and 2 integrons

The presence or absence of class 1 and 2 integrons were determined using PCR. Specifically, detection of the *intI1* and *IntI2* genes encoding integrases of class 1 and class 2 integrons, respectively, was assayed by using specific primers (Table 3) as previously described (Mazel et al., 2010).

2.3.3 *Agarose gel electrophoresis*

PCR-amplified fragments were analysed by electrophoresis in agarose gels (2% in 0.5 x TBE buffer) containing 0.5 µl/ml ethidium bromide. The PCR products (15 µl) were mixed with 5 µl of TBE buffer shortly before loading into the gels. DNA bands were visualized and photographed under UV illumination.

Table 3. Primer sets and integron amplified fragments

Genes/primer sequence (5'→3')	Size
IntI 1 (class 1 integrase) GGGTCAAGGATCTGGATTTCG ACATGGGTGTAAATCATCGTC	**483 bp**
IntI 2 (class 2 integrase) CACGGATATGCGACAAAAAGGT GTAGCAAACGAGTGACGAAATG	**788 bp**

3. Results and Discussion

3.1 Prevalence of *E. Coli* in lettuce

We used the microbiological criteria implemented in the case of vegetable products according to the French ministerial rules and guidelines from May 28, 1997. These concern criteria applicable to raw, peeled and sliced vegetables as well as to ready-to-eat raw vegetables. Using these guidelines, we found that among 100 specimens analysed, 80% were of satisfactory quality, whereas 10% were acceptable for consumption and the remaining 10% were of unacceptable/non-satisfactory microbiological quality. This percentage of *E. coli* contamination is quite high as compared to findings by others. Indeed, Johannessen et al. (2002) assessed the bacteriological quality and potential health risk of 890 fresh and pre-sliced lettuce heads from Norwegian vegetable markets. The authors found the prevalence of thermo-tolerant coliforms to be 2.5%. Similarly, Meldrum et al. (2009), showed that among 1213 lettuce and vegetable specimens, 4.7% are of non-satisfactory bacteriological quality due to the presence of *E. coli*, with cucumbers being the most contaminated (6%).

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However, in a study carried out from September 1999 to Mars 2000, Soriano et al. (2001) analysed 370 specimens from 20 restaurants in Valencia (Spain). The authors compared batches of unwashed lettuce with others that were pre-washed in water containing sodium hypochlorite. They found high prevalence (10%) of *E. coli* in unwashed specimens. The authors' findings concur with earlier studies by others (King et al., 1991) who found low prevalence of *E. coli* in washed/disinfected lettuce as compared to unwashed specimens. Thus, *E. coli* contamination could be used as a reliable indication of contamination during bacteriological assessments of crude products.

3.2 Antibiotic susceptibitity testings reveal resistance to several antibiotics

We carried out phenotypic tests for antibiotic resistance of 27 randomly selected *E. coli* isolates. We found that 17 isolates (63%) were sensitive to all antibiotics tested. Importantly however, we found that 10 isolates (37%) were resistant to antibiotics used in treatments of *E. coli* infections.
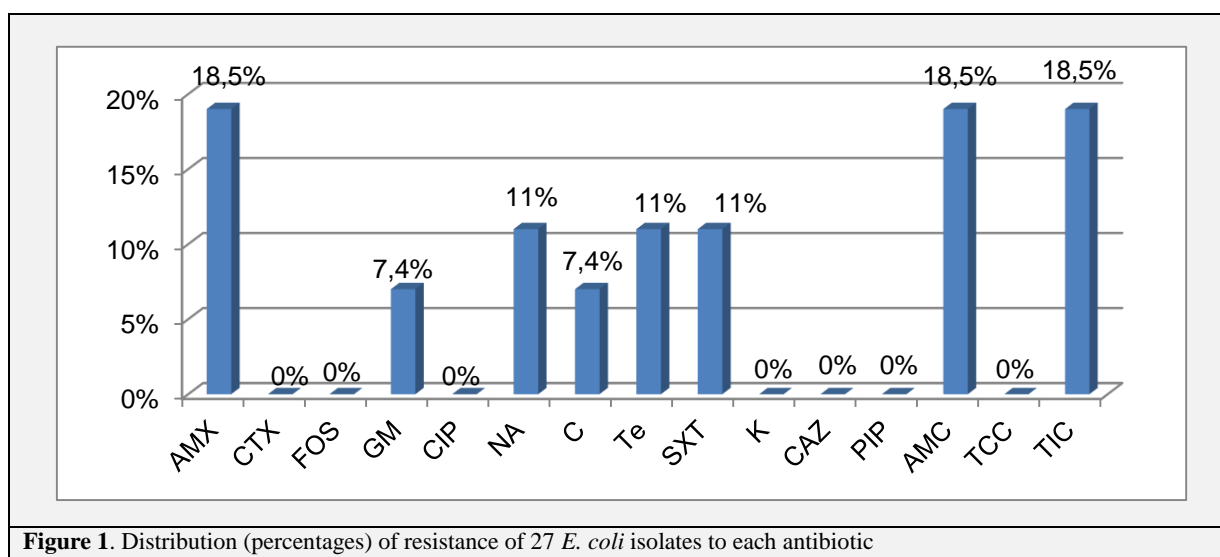

Figure 1. Distribution (percentages) of resistance of 27 *E. coli* isolates to each antibiotic

Specifically, for each antibiotic tested (Fig. 1), none of the isolates studied were resistant to cefotaxime (CTX), piperacillin (PIP), ceftazidime (CAZ; a third-generation cephalosporin), ciprofloxacin (CIP), fosfomycin (FOS), kanamycin (K) and to the combination of Ticarcillin and clavulanic acid (TCC). The resistant isolates exhibited different profiles as follows: First, five isolates (18.5%) were resistant to three antibiotics, namely Amoxicillin without clavulanic acid (AMX), Amoxillin with clavulanic acid (AMC) and Ticarcillin (TIC). Second, three isolates (11.11%) were resistant to each of three other antibiotics, including Tetracyclines (Te), Nalidixic acid (NA) and Trimethoprim/Sulfamethoxazole (SXT). These latter 3 isolates exhibited hyperproduction of cephalo-sporinases. Finally, two isolates (7.4%) were found to be resistant to each of yet two other different antibiotics, including Chloramphenicol (C) and Gentamicin GM).

The entire collection of our isolates was unable to produce extended-spectrum β-lactamases. Resistance to amoxicillin is usually attributed to production of low- or high-level penicillinase (Caroff et al., 2000; Jorgensen et al., 2010).

Three (≈ 11%) of our isolates were resistant towards nalidixic acid, a quinolone. Resistance to quinolones is generally caused by mutations in specific regions of the genes encoding DNA gyrase A or topoisomerase IV. However, recent evidence points to the emergence of involvement of transmissible plasmid-mediated resistance to quinolones (Strahilevitz et al., 2009).

The nalidixic acid-resistant 3 isolates were also resistant to tetracyclines as well as to the combination Trimethoprim/Sulfamethoxazole. This low percentage (≈ 11%) is at odds with the high percentages of *E. coli* detected in human and animal sources in Tunisia and worldwide (Van et al., 2007; Meyer et al., 2008; Soufi et al., 2009). Nevertheless, tetracycline resistance in our isolates is alarming in a developing country, as it may reflect contamination of raw vegetables through contaminated irrigation water and manure.

We observed resistance to chloramphenicol and gentamicin only in two isolates (7.4%). These findings are in agreement with previous studies (Jouini et al., 2007; Soufi et al., 2009, 2011) showing low levels of resistance to these antibiotics.
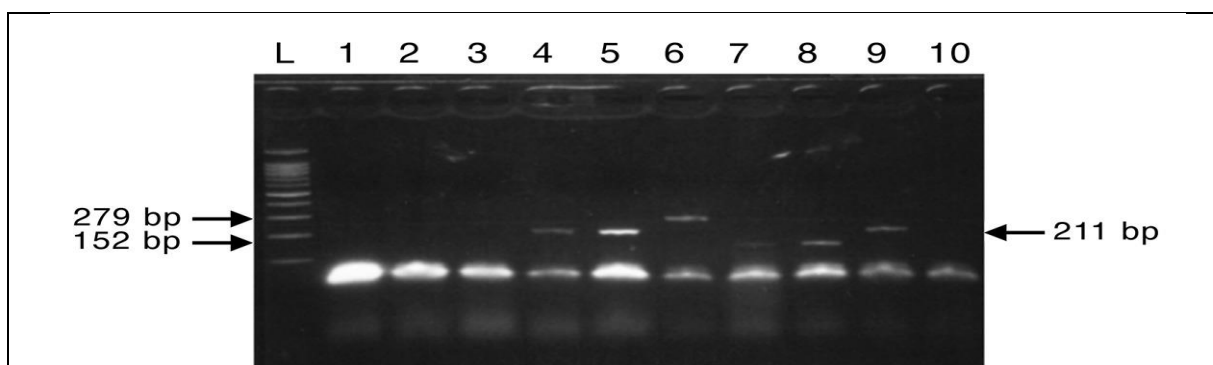
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Figure 2. Representative image (gel 1) of PCR profiles for the phylogenetic groups of *E. coli* isolates. L, 100 bp DNA ladder. Lanes 1, 2, 3, and 10, group A (A_o). Lanes 4, 5 and 9, group A (A_1). Lanes 7 and 8, group B1. Lane 6, group D (D_1).
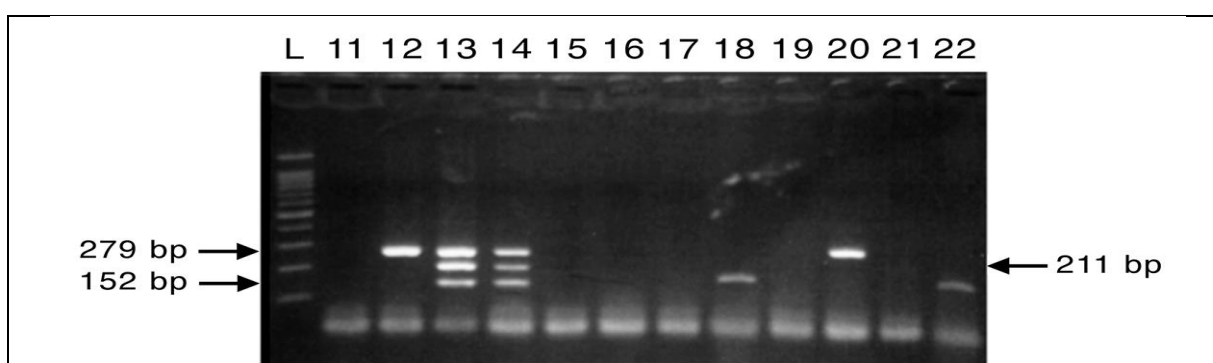

Figure 3. Representative image (gel 2) of PCR profiles for the phylogenetic groups of *E. coli* isolates. L, 100 bp DNA ladder. Lanes 11, 15, 16, 17, 19 and 21 portray group A (A_0). Lane 18, group A (A_1). Lane 22, group B1. Lanes 13 and 14, group B2 ($B2_3$). Lanes 12 and 20, group D (D_1).

3.3 Predominance of *E.coli* phylogenetic groups A and B1 in the isolate collection

For further characterization of the 27 *E. coli* isolates selected from raw lettuce, we carried out assays to determine their phylogenetic groups and subgroups. Representative images of the analyses of PCR products carried out to determine the distibution of the phylogenetic groups and subgroups (presence or absence of the genes *ChuA*, *YjaA* and *TspE4C2*) in *E. coli* isolates are shown in Figures 2 and 3.

We found that 16 isolates (59.3%) belonged to group A, among these 12 (44.5%) and 4 (14.8%) isolates were of the subgroups A_o and A_1, respectiveley. Four isolates (14.8%) segregated into group/subgroup B1. Two isolates (7.4%) fell into group B2, none (0%) of which were of subgroup $B2_2$, whereas the two segregated into subgroup $B2_3$. Finally, 5 isolates (18.5%) belonged to group D, among these 4 isolates (14.8%) segregated in subgroup D1 and 1 isolate (3.7%) was categorized as subgroup D2. The distribution of the phylogenetic groups of the *E. coli* isolates is summarized in Table 4.

From our data, it emerges that *E. coli* strains of the phylogenetic groups A and B1 were predominant as they represented 74% (20 isolates) of our isolates, whereas groups B2 and D were under-represented since only 7 isolates (26%) were assigned to these groups. Overall, our findings echo those described in previous studies where groups A, B1 and D were found to be more prominent than group B2 (Ho et al., 2009). It is well-established that *E. coli* strains belonging to the B2 group are the most virulent, followed by group D strains, contrasting with those of group A and B1, which are commensal strains (Bonacorsi et al., 2005; Ewerset et al., 2007). Hence, we can conclude that 26% of our isolates are potentially virulent.

Table 4. Distribution of *E. coli* phylogenetic groups and subgroups among 27 isolates

Isolate	ChuA	YjaA	TspE4C2	Phylogenetic group	Subgroup

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N°	(279pb)	(211pb)	(152pb)		
1	–	–	–	A	A_0
2	–	–	–	A	A_0
3	–	–	–	A	A_0
4	–	+	–	A	A_1
5	–	+	–	A	A_1
6	+	–	–	D	D_1
7	–	–	+	B1	B1
8	–	–	+	B1	B1
9	–	+	–	A	A_1
10	–	–	–	A	A_0
11	–	–	–	A	A_0
12	+	–	–	D	D_1
13	+	+	+	B2	$B2_3$
14	+	+	+	B2	$B2_3$
15	–	–	–	A	A_0
16	–	–	–	A	A_0
17	–	–	–	A	A_0
18	–	+	–	A	A_1
19	–	–	–	A	A_0
20	+	–	–	D	D_1
21	–	–	–	A	A_0
22	–	–	+	B1	B1
23	–	–	–	A	A_0
24	+	–	+	D	D_2
25	–	–	+	B1	B1
26	–	–	–	A	A_0
27	+	–	–	D	D_1

3.4. Positive correlation between the presence of integrons and resistance to antibiotics

It is well-established that bacterial resistance to antibiotics is a major problem in human medicine as it thwarts the treatment of infectious diseases. This is even further compounded by the increasing emergence of bacterial strains exhibiting multi-drug-resistance (Hue et al., 2010). Dissemination of antibiotic resistance in *Enterobacteriaceae* is generally due to expression of resistance genes located on integrons or transposons, which can be chromosomal or plasmidic. Integrons provide a genetic basis for capture and expression of resistance genes as they can harbour 1 to 8 resistance genes (Gillings et al., 2008).
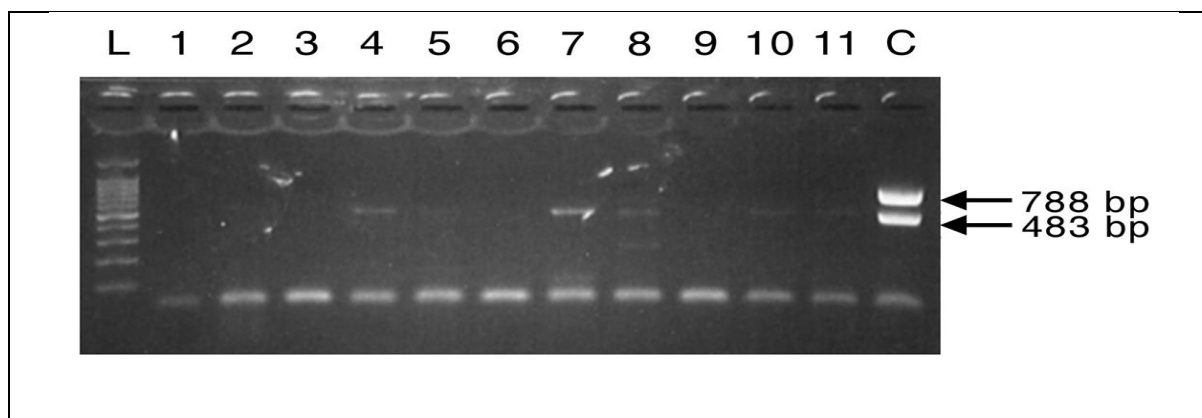

Figure 4. Representative image of PCR profiles specific to the *IntI 1* and *IntI 2* genes. L, 100 bp ladder. C, positive control isolate showing the presence of class 1 and class 2 integrons. Lanes 2, 4, 5, 7, 8, 10 and 11, isolates with class 1 integron. Lanes 1, 3, 6 and 9, isolates free of integrons.

Among the 27 isolates studied, PCR assays (representative data of these experiments are shown in Fig. 4) indicated that 14 isolates (51.8%) harbour class 1 integron, whereas class 2 integron was not detected. This emphasizes the predominance of class 1 integrons over that of class 2. Furthermore, we found that 13 isolates (48.15%) were devoid of integrons. Our findings are similar to those of others (Soufi et *al.*, 2009, 2011; Domingues et *al.*, 2012). In this regard, class 1 integrons are genetically active entities as they are able to capture and express gene cassettes encoding antibiotic resistance factors, whereas class 2 integrons are stable units and unable to integrate new gene cassettes.

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By providing a global view of resistance to antibiotics combined with determination of the presence/absence of integrons in 27 isolates (Table 5), we were able to reveal a positive correlation between antibiotic resistance and presence of integrons. The ten *E. Coli* isolates (37%; isolates 2, 4, 5, 7, 8, 10, 11, 19, 20 and 27) that displayed resistance to different antibiotics all harboured class 1 integrons, further emphasizing the crucial role of integrons in dissemination and acquisition of resistance genes. Remarkably, 4 isolates were sensitive to antibiotics, yet carried class 1 integrons, suggesting the presence of a genetic pool for potential capture of resistance genes and a likelihood of presence of empty integrons, as shown in previous studies (Quiroga et *al*., 2013; Rowe-Magnus et *al*., 1999).

Table 5. Summary of the findings in the 27 *E. coli* isolates

Isolate	Phylogenetic group	Subgroups	Resistance profile	Integrons
E. coli 1	A	A_0	Sensitive	Absent
E. coli 2	A	A_0	Resistant to (AMX), (TIC) and (AMC)	Presence of class 1 integron
E. coli 3	A	A_0	Sensitive	Absent
E. coli 4	A	A_1	Resistant to (AMX), (TIC) and (AMC)	Presence of class 1 integron
E. coli 5	A	A_1	Resistant to (AMX), (TIC) and (AMC)	Presence of class 1 integron
E. coli 6	D	D_1	Sensitive	Absent
E. coli 7	B1	B1	Resistant to (Te), (NA) and (SXT)	Presence of class 1 integron
E. coli 8	B1	B1	Resistant to (Te), (NA) and (SXT)	Presence of class 1 integron
E. coli 9	A	A_1	Sensitive	Absent
E. coli 10	A	A_0	Resistant to (C) and (GM)	Presence of class 1 integron
E. coli 11	A	A_0	Resistant to (Te), (NA) and (SXT)	Presence of class 1 integron
E. coli 12	D	D_1	Sensitive	Absent
E. coli 13	B2	$B2_3$	Sensitive	Absent
E. coli 14	B2	$B2_3$	Sensitive	Absent
E. coli 15	A	A_0	Sensitive	Absent
E. coli 16	A	A_0	Sensitive	Absent
E. coli 17	A	A_0	Sensitive	Absent
E. coli 18	A	A_1	Sensitive	Absent
E. coli 19	A	A_0	Resistant to (AMX), (TIC) and (AMC)	Presence of class 1 integron
E. coli 20	D	D_1	Resistant to (AMX), (TIC) and (AMC)	Presence of class 1 integron
E. coli 21	A	A_0	Sensitive	Presence of class 1 integron
E. coli 22	B1	B1	Sensitive	Presence of class 1 integron
E. coli 23	A	A_0	Sensitive	Presence of class 1 integron
E. coli 24	D	D_2	Sensitive	Presence of class 1 integron
E. coli 25	B1	B1	Sensitive	Absent
E. coli 26	A	A_0	Sensitive	Absent
E. coli 27	D	D_1	Resistant to (C) and (GM)	Presence of class 1 integron

4. Conclusion

The presence of *E. coli* in food products, and notably in raw vegetables, constitutes a potential risk for food illness outbreaks, especially in hospitals where food is prepared on a large scale. In this context, the most alarming is the fact that raw vegetables are served to vulnerable patients who are already

weakened by other diseases, advanced age, or subsequent to various invasive surgeries. This indeed constitute a major public health issue.

E. coli is an endogenous, benign bacteria found normally among the intestinal microflora. However, pathogenic strains occasionally induce intestinal and extra-intestinal infections. *E. coli* belongs to the coliform group of thermo-tolerant bacteria, able to survive and grow at temperatures reaching 44 °C. Furthermore, *E. coli* is an excellent "reporter bacteria", indicating fecal contamination of raw vegetables.

We show that 10% of lettuce heads analysed were contaminated. This level is relatively high when compared to previous findings by others. Despite low levels of resistance to antibiotics in our *E. coli* isolates, it would be necessary to establish a program to control antibiotic resistance in laboratories in Tunisia. Resistance to different antibiotics was of 37%. The distribution of resistance among these includes, 18.5% towards AMX, TIC and AMC, 11.11% to Te, NA and SXT, as well as 7.4% to C and GM.

The majority of our isolates (74%) segregated into the phylogenetic groups A and B1, known to include commensal strains. However, this result does not totally exclude pathogenicity of these isolates.

The predominant existence of class 1 integrons detected in 51.8% of the isolates over that of class 2 integrons, which was not detected, suggests that these are endowed with a powerful genetic basis for capture and expression of resistant genes, entailing further resistance gene dissemination. The 37% isolates that were resistant to different antibiotics, all contained class 1 integrons, further strengthening the role of these genetic entities in the spread and acquisition of antibiotic resistance genes.

Our data showing the presence of relatively high levels of *E. coli* displaying a range of potential threatening characteristics, combined with the fact that the isolates were from raw lettuce consumed by patients and personnel in a hospital, emphasize the urgent need for the establishment and implementation of control programs, such as the Hazard Analysis & Critical Control Points (H.A.C.C.P), together with improving public awareness. These are necessary measures to prevent catastrophic outbreaks.

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