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### Abstract

Raw milk has recently been reported as a source of extended-spectrum beta-lactamase (ESBL) and carbapenemase genes. We thus investigated the prevalence of ESBL- and carbapenemase-producing *Enterobacteriaceae* in raw milk in Lebanon in order to assess the risk of transfer of these bacteria to humans. A high prevalence (30.2 %) of CTX-M-15-producing *K. pneumoniae* was detected in raw bovine milk. Three main *K. pneumoniae* clones were identified by PFGE and MLST typing. Southern blot experiments revealed that one of these clones carried the *blaCTX-M-15* gene chromosomally. Moreover, one OXA-48-producing *K. pneumoniae* ST530 and seven CTX-M-15-producing *Escherichia coli* sharing the same ST were also detected. These findings highlight the spread of dominant CTX-M-15-producing *K. pneumoniae* clones and OXA-48-producing isolates in the food chain. Milk, which is mostly consumed raw in Lebanon, may be a source of human exposure to ESBLs and carbapenemases.

Extended-spectrum beta-lactamases (ESBLs) and carbapenemases are a major public health threat since these enzymes impair the treatment of human infections caused by *Enterobacteriaceae* resistant to the last generations of beta-lactams. ESBLs are widely disseminated not only among humans but also in animal populations and the environment, making their global control a major challenge. In contrast, carbapenemases are still mainly hospital-associated, but certain enzymes such as OXA-48 have also spread throughout the community in numerous countries [1–3]. In Lebanon, the occurrence of OXA-48 in a large diversity of strains in both hospital and community settings suggests an endemic situation [2]. Carbapenemase-producing bacteria have rarely been reported in animals, most likely as a result of the absence of carbapenem use in veterinary medicine. Therefore, their recent emergence in animals raises concerns, in particular with regard to the risk of transfer to humans via the food chain.

There is abundant scientific evidence that food products can be contaminated by ESBLs, thereby suggesting the potential for transfer of ESBL-producing bacteria or genes to humans through food consumption and/or handling. For example, chicken meat is regarded as one of the main contributors in the transmission of ESBLs to humans, but other sources such as vegetables or ready-to-eat food have also been incriminated [4–6]. Raw milk was also recently reported as a source of ESBL and carbapenemase genes, and this should be considered a crucial and still underestimated issue in terms of human contamination in countries where milk is principally consumed raw. Indeed, ESBL-producing *Enterobacteriaceae* were detected in 82/866 (9.5 %) and 8/80 (10 %) of raw bulk tank milk samples in Germany and Indonesia, respectively [7, 8]. Milk samples collected from individual cows were also found to harbour ESBL-producing *E. coli* (9/73, 12.3 %) or *Klebsiella pneumoniae* (2/129, 1.5 %) in India, by ESBL-producing *E. coli* in the Czech Republic (2/263, 0.7 %) and by NDM-5-producing *E. coli* in Algeria [9–12].

In Lebanon, recent studies reported a high prevalence of faecal carriage of CTX-M-15-producing *E. coli* in dairy cows, and the emergence of OXA-48 carbapenemase in poultry production [13, 14]. These data suggest that food animals and foodstuffs may constitute an important reservoir of ESBL and carbapenemase genes in Lebanon. In regard to these data, we deemed it important to assess the potential risk of ESBL and carbapenemase gene

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**Abbreviations:** ESBL, extended-spectrum beta-lactamase; MALDI-ToF, matrix assisted laser desorption ionisation - time of flight; MLST, multi-locus sequence typing; PBRT, PCR-based replicon typing; PFGE, pulsed-field gel electrophoresis; ST, sequence type.
transmission to humans through milk consumption, a common food product that is usually not subjected to heat treatment in this country. Our goal was thus to determine the prevalence of ESBL- and/or carbapenemase-producing Enterobacteriaceae in raw milk and to characterize the collected isolates molecularly, in order to help clarify risk assessment issues in terms of potential human transfer through milk consumption.

Milk samples from 154 healthy adult cattle were aseptically collected from 63 different farms located in North Lebanon between September and November 2015. Enterobacteriaceae resistant to broad-spectrum cephalosporins were selected by directly plating 100 µl of milk on MacConkey agar plates supplemented with ceftazidime (4 mg l\(^{-1}\)) or meropenem (2 mg l\(^{-1}\)). Of the 63 farms sampled, 18 (28.6 %) were positive for ESBL-producing Enterobacteriaceae and one (1.6 %) was positive for ESBL- and carbapenemase-producing K. pneumoniae. All farms were small (from one to eight animals) with no cross-interactions, and cows were milked by hand. Forty-three ESBL-producing Enterobacteriaceae (27.9 %), identified as K. pneumoniae (n=36, 23.4 %) and E. coli (n=7, 4.5 %) using MALDI-TOF MS (Microflex, Bruker), were isolated from the 154 milk samples. ESBL-producing K. pneumoniae were found to be highly dominant and identified for all 19 positive farms compared to ESBL-producing E. coli, recovered from five farms only. This is far the highest prevalence of ESBLs in raw milk yet reported [7–11], possibly resulting from the uncontrollable use of antimicrobials in animals. The dominance of ESBL-producing K. pneumoniae was unexpected as K. pneumoniae is not reported as a common pathogen in dairy cattle compared to humans. Since milk is largely consumed raw in Lebanon, these data led us to anticipate a major risk of transfer of ESBL-producing K. pneumoniae to humans through milk consumption.

The 36 ESBL-producing K. pneumoniae clustered in three major sequence types: ST219 (n=21, collected from 15 farms), ST17 (n=10, from three farms) and ST429 (n=5, from five farms) (Fig. 1). Except for one isolate each from clusters ST219 and ST17, all XbaI-Pulsed Field Gel Electrophoresis (PFGE) patterns within a given ST shared >90 % similarity (Fig. 1). The unique carbapenemase-producing K. pneumoniae was the isolate belonging to ST530. This highlights the epidemic spread of a limited number of ESBL-producing K. pneumoniae clones across unrelated farms, also exemplified by the detection of ST219 and ST17 from several cows within the same farm. Several hypotheses may be proposed to explain this epidemiology. The prevalence of K. pneumoniae carriage, which has been linked to poor hygiene of the animals’ legs and udder, might be underestimated [15]. The converse would suggest human contamination since all cows were milked by hand, thus indicating a high prevalence of these K. pneumoniae clones in the community. To date, K. pneumoniae ST219, ST429 and ST530 have been sporadically reported in humans while the CTX-M-15-producing ST17 clone is more widely disseminated in China, the USA and Europe, and its capacity to cause outbreaks and subsequently persist in the digestive tract is documented [16–19]. Unfortunately, whereas high rates of ESBL-producing K. pneumoniae were reported in Lebanese hospitals (~30 %) [20], no data are available on the population structure of K. pneumoniae and its dissemination in the community. Of note, the seven ESBL-producing E. coli originated from five different farms but clustered in a new ST (allelic profile 526/7/1/1/8/6), close to ST5920. This also suggests a clonal spread in cattle, though at a smaller scale than that observed for K. pneumoniae.

All ESBL-producing K. pneumoniae and E. coli harboured a \(\text{bla}_{\text{CTX-M-15}}\) gene, as detected using previously published PCRs [21, 22]. Among K. pneumoniae isolates, four additionally displayed the \(\text{bla}_{\text{SHV-12}}\) gene and one harboured the \(\text{bla}_{\text{OXA-48}}\) gene on an IS1999.2 transposon. To the best of our knowledge, this is the second worldwide report of carbapenemase-producing Enterobacteriaceae in raw milk; the first identified NDM-5-producing E. coli isolates in Algeria [12]. The current study is also the second report of OXA-48 in the food sector in Lebanon, following the recently described OXA-48-encoding E. coli ST38 in a healthy fowl [14]. This shows that, in this country, the OXA-48 enzyme is spreading not only through E. coli but also K. pneumoniae in the food chain.

Using the PCR-based replicon typing (PBRT) scheme (Diatheva, Fano, Italy) and Southern blots on S1-PFGE gels, the \(\text{bla}_{\text{OXA-48}}\) gene was shown to be located on a prototypic IncF plasmid. Likewise, the \(\text{bla}_{\text{CTX-M-15}}\) gene was shown to be located on IncF plasmids in E. coli and on IncFIIK plasmids in the ST17 and ST530 OXA-48-producing K. pneumoniae clones. IncF plasmids have been widely reported to disseminate \(\text{bla}_{\text{CTX-M-15}}\) in Enterobacteriaceae from both animal and human origin, and obviously also play a major role in the spread of this ESBL gene in Lebanon [23–25]. On the contrary, PFGE on 16S-rDNA digested DNA detected by Southern blot showed that \(\text{bla}_{\text{CTX-M-15}}\) was chromosomally encoded in the 21 isolates belonging to ST219, which is a much less frequent situation compared to the \(\text{bla}_{\text{CTX-M-15}}\) location on plasmids [26].

In conclusion, this study demonstrates the epidemic spread of K. pneumoniae clones in milk from unrelated farms located in a geographic area of Lebanon covering a quarter of the whole country, as well as the detection of an OXA-48-producing K. pneumoniae isolate. An epidemiological link between these K. pneumoniae clones recovered from milk and those responsible for human infections in Lebanon is plausible. On the other hand, no data are available on the population structure of K. pneumoniae in clinics in Lebanon. Indeed, a limitation of this study is the absence of samples taken either from the environment of the farms or from people in close contact with the milking cows; this would have helped us elaborate hypotheses on the origin and transfer of the successful K. pneumoniae clones. Taking all the above results together, this study emphasizes that surveillance of ESBL- and carbapenemase-producing
Enterobacteriaceae in food animals and foodstuffs in Lebanon is now a priority, and is to be compared to human epidemiology from a One Health perspective. Indeed, the presence of such pathogens is of public health concern, not only from the clinical perspective but also in consideration of the risk of silent dissemination of the organisms or the corresponding genes within a healthy population.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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