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Prevalence of asymptomatic carriers of Shiga toxin-producing *Escherichia coli* (STEC) in dairy cattle farms in the governorate of Blida (Algeria)

Djamila Baazize-Ammi[1, 2], Ouahiba Gassem[3], Fawzi Derrar[4], Kahina Izri[4], Mohamed Brahim-Errahmani[5], Jean Gagnon[6], Djamel Guetarni[3], Yahia Cheblounе[6]

[1]Veterinary Institute, [3]Faculty of Nature and Life Science, [5]Faculty of Sciences, University of Blida 1, Algeria
[2]Veterinary Institute, University of Tarf, Algeria
[4]Respiratory Virus Unit, Virology Department, Pasteur Institute of Algeria, Algeria
[6]Pathogenesis and Lentiviral Vaccination, PAVAL Laboratory, Joseph Fourier University Grenoble 1, Grenoble Cedex 9, France
ychebloune@lyon.inra.fr

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Abstract

We investigated whether dairy beef cattle raised in Algeria are Shiga toxin-producing *Escherichia coli* (STEC) carriers. *Stx*1 and *stx*2 genes were analysed in DNA isolated from 200 faecal samples collected from adult dairy cows from 27 randomly selected farms in Blida, North Algeria, after amplification by PCR. Samples from 61 (30.5%) animals out of the 200 were positive and were located in 18 farms with a prevalence of 66.7%. Interestingly, no sample from any cow was positive for only the *stx*2 gene, while in contrast, samples from 51 cows were positive for the *stx*1 gene alone (83.6%) and those from 10 other cows were positive for both *stx*1 and *stx*2 genes (16.4%). It should be noted that the faecal samples infected with pathogens carrying the two genes originated from 4 out of the 18 farms that were found to be positive, with a rate of 22.2%.

Keywords: cattle, *Eschrichia coli*, Shiga toxins, faeces, *stx* genes, PCR, Algeria.

Introduction

The Shiga toxin-producing *Escherichia coli* (STEC) are bacteria that are currently being considered important emerging pathogens threatening public health. They have been known since 1982, when *E. coli* O157:H7 was isolated for the first time during the outbreaks of haemorrhagic colitis in the United States due to the consumption of contaminated hamburgers (24). According to the Centers for Disease Control (CDC), STEC are responsible for at least 70 000 infections a year in the USA. Since the emergence of STEC, these bacteria have been involved in several outbreaks or sporadic cases worldwide (Canada, Japan, Africa, and Europe) as a result of the consumption of contaminated animal products (minced meat, milk, and raw milk cheese products) (13, 31). To date, over 100 different serotypes of STEC have been identified, all causing bacterial gastroenteritis in humans and some known to induce haemorrhagic colitis and haemolytic uremic syndrome (HUS) (10). Numerous studies have reported that STEC-related illness is not always associated with fever and haemorrhagic colitis and is not restricted to a particular season (16). Recently, a large outbreak of HUS caused by this bacteria (O104:H4) occurred in Europe, affecting more than 4000 patients with diarrhoeal disease and resulting in the death of about 54 patients (4, 11). What resulted from this outbreak raised the question of the emergence of new types of non-O157 STEC with similar or increased virulence compared to O157:H7 strains (4, 11).

The epidemiology of STEC infections has evolved. Consumption of fruit and vegetables,

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ingestion of contaminated water, contact with animals and their environment as well as inter-human transmission are other means of spreading these infections to man (14). The O157:H7 serotype is the one most commonly found in clinical practice, but other serogroups are also increasingly involved in outbreaks. It was demonstrated that the pathogenic STEC strains belong to serotypes O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7 (3). The pathogenicity of STEC is being attributed to the products of *stx*1 and *stx*2 genes encoding Shiga toxins (1 and 2) and to the *eae* gene encoding intimin. The combination of these genes would enhance the risk and severity of the infections (6). Studies suggest that the strains carrying *stx*2 gene only are more virulent than strains carrying *stx*1 gene only or both *stx*1 and *stx*2 genes (6).

STEC were isolated from a large number of animal species, but cattle are the main reservoir. Those carrying and excreting STEC are most often asymptomatic whenever they host the bacteria in their digestive tract; hence they are called healthy carriers (12, 15). Although STEC infection in human is a common cause of bloody diarrhoea in developed countries and causes many outbreaks, the incidence of this bacterial infection in North African countries such as Algeria is not clear and has not been well studied. In the Maghreb, more specifically in Algeria, the amount of STEC carriage by cattle and other animals used in the food industry is unknown. Because these bacteria have not been investigated at the local level, their involvement, in terms of being pathogenic agents, passes unnoticed. Only few works have reported the presence of this type of bacteria in Algerian foodstuffs (2, 8). With the fast evolution of the society towards the growing food processing industry, consumption of fast food products and increased consumption of cattle meat and milk products, this bacterial infection in cattle should be monitored to learn how to set up the best sanitary measures that will minimise outbreaks in consumers.

The aim of this study was to explore the asymptomatic occurrence of STEC in dairy cattle farms in the governorate of Blida by searching for the common sequence of *stx*1 and *stx*2 genes and the specific sequences of *stx*1 gene and *stx*2 gene by PCR.

Material and Methods

Samples. This study, performed between May 2011 and June 2012, investigated 200 samples of faeces collected from adult dairy cows belonging to 27 farms from districts of the governorate of Blida, Algeria (Fig. 1). The samples were statistically chosen taking into consideration (i) the numbers of animals in each district covered by the study, (ii) following a 1/10 sampling mode, and (iii) with random selection of farms, considering the minimal herd to comprise at least five cows. The sampled animals were mainly of Holstein breed (72%) while the remaining cows were Montbéliarde, Fleckvieh, and Simmental. Most cows were between three and five years (73%) of age or over six years (27%). Although the health status of the studied animals was not examined in depth, neither diarrhoea nor indigestion was observed when the faecal samples were taken.

The fresh faeces, sampled from the rectum using the classical procedures, were collected into labelled and tagged sterile jars and transported in refrigerated containers to the laboratory for processing. The technical conditions of DNA extraction and PCR analysis were first performed at the PAVAL Laboratory, Grenoble, and then transferred to the Viral Respiratory Unit of the Pasteur Institute, Algeria.

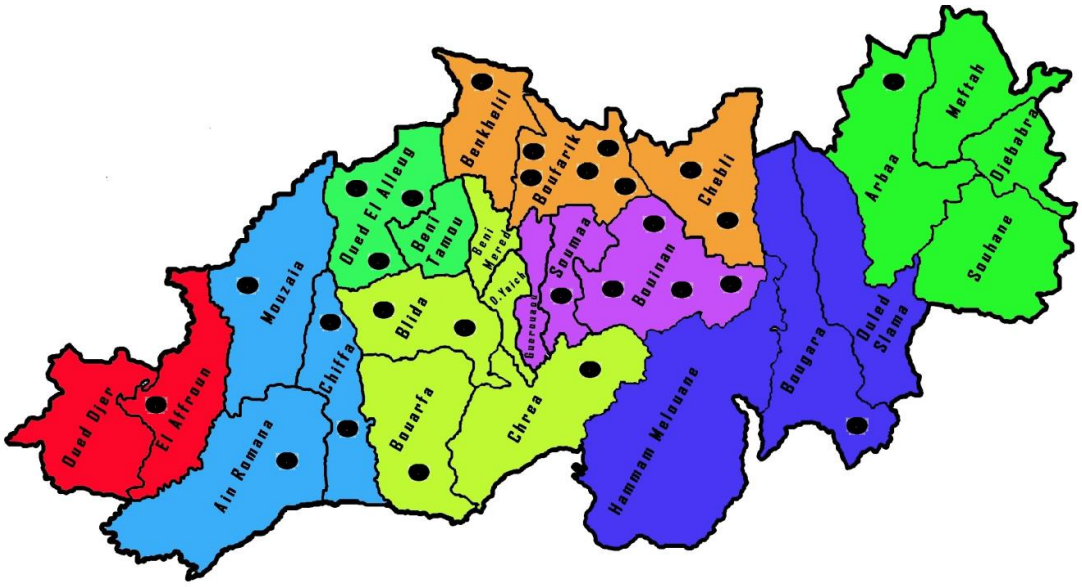

Fig. 1. Geographic map of the districts of the governorate of Blida showing the distribution of the herds. The black dots represent the localisation of the dairy cow herds in each district

Table 1. Nucleotide sequences of the primers used for PCR

Virulence factor	Code of primers	Sequence (5'-3')	Size of PCR product (bp)	References
*stx*1 and *stx*2	VT com-u	GAGCGAAATAATTTATATGTG	518	(33)
	VT com-d	TGATGATGGCAATTCAGTAT		(18)
*stx*1	B54	AGAGCGATGTTACGGTTTG	388	(9)
	B55	TTGCCCCCAGAGTGGATG		
*stx*2	B56	TGGGTTTTTCTTCGGTATC	807	(7)
	B57	GACATTCTGGACTCTCTT		

Sample processing and extraction. The faecal samples (25 g) were resuspended in 100 mL of phosphate buffered saline (PBS, GIBCO Invitrogen, USA), pH 7.4, homogenised, and filtered through sterile hydrophilic gas and a 4 mm Whatman paper. The filtrate was centrifuged at 6000 rpm for 10 min at 4°C and then the pellet was washed three times with PBS, pH 7.4. The resulting pellet was frozen for DNA extraction, which was performed using the QIAamp® DNA Stool (Qiagen, USA) according to the manufacturer's instructions.

PCR. The approach adopted was based on the search for the common sequence of *stx*1/*stx*2 genes, as a screening test, subsequently followed by the search for the specific sequences of *stx*1 and *stx*2 genes on positive samples. The GoTaq Flexi DNA Polymerase kit (Promega, USA) and oligonucleotide primers reported in Table 1 (Eurogentec, France) were used. The PCR mix contained: 10 µL of 5x Green or Colorless GoTaq Flexi Buffer (1x); 3 µL of $MgCl_2$ (1.5 mM); 1 µL of dNTP (0.2 mM); 1 µL of primer 1 (0.4 µM); 1 µL of primer 2 (0.4 µM); 23.70 µL of sterile distilled water; and 0.3 µL of Taq polymerase (GoTaq DNA Polymerase, 5 U/µL). This mix was added to 10 µL of DNA extracted from each faecal sample.

The amplification was performed in a Mastercycler gradient thermocycler (Eppendorf, Germany) according to the following programme: initial denaturation at 94°C for 3 min, 35 cycles (each including denaturation at 94°C for 90 sec, hybridisation at 59°C for 90 s, and elongation at 72°C for 120 s), followed by a final extension step performed at 72°C for 5 min as previously described (19). The PCR products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide and observed under UV light using a transilluminator (Gel Documentation system XR, BIO-RAD, USA). The size of the fragments was determined by the migration of a molecular weight marker (Promega, USA) from 100 to 1500 pb.

Results

In order to identify the STEC-carrying animals, the PCR products from all studied samples were separated in agarose gels and specific bands detected following ethidium bromide staining. The presence of a band at 518 bp corresponding to the sequence common to both the *stx*1 and *stx*2 genes was demonstrated (Fig. 2a). Another band at 388 bp, corresponding to the sequence of *stx*1 gene, was also detected in all samples containing the common sequence (Fig. 2b) while in contrast the band of 807 bp, corresponding to the sequence of *stx*2 gene, was only identified in a few samples (Fig. 2c).

The summary of the results of the electrophoretic analyses is shown in Table 2. The presence of the *stx*1/*stx*2 common sequence to both genes was successfully confirmed in 61 out of the 200 samples (30.5%). Interestingly, the presence of the *stx*1 gene was found in all positive samples for the common sequence (100%). In contrast, however, the band corresponding to the *stx*2 gene was detected in only 10 out of the 61 samples (16.4%). These results demonstrated that samples from animals in 18 farms were positive for STEC, thereby qualifying those herds as potential STEC-carrier cows (66.7%). Therefore, the carriage rate of STEC ranged from 4.2% to 100%.

The distribution of individual *stx*1 and *stx*2 genes amongst the 61 positive animals and herds is presented in Table 3. It indicates that 51 out of the 61 cows infected with STEC carry only the *stx*1 gene (83.6%). These animals were distributed over 14 (77.8%) farms. Only ten cows were found to carry both *stx*1 and *stx*2 genes (16.4%). These animals were distributed over four farms, with an incidence of 22.2%. Interestingly, our study failed to find any animal that carried STEC with only the *stx*2 gene.

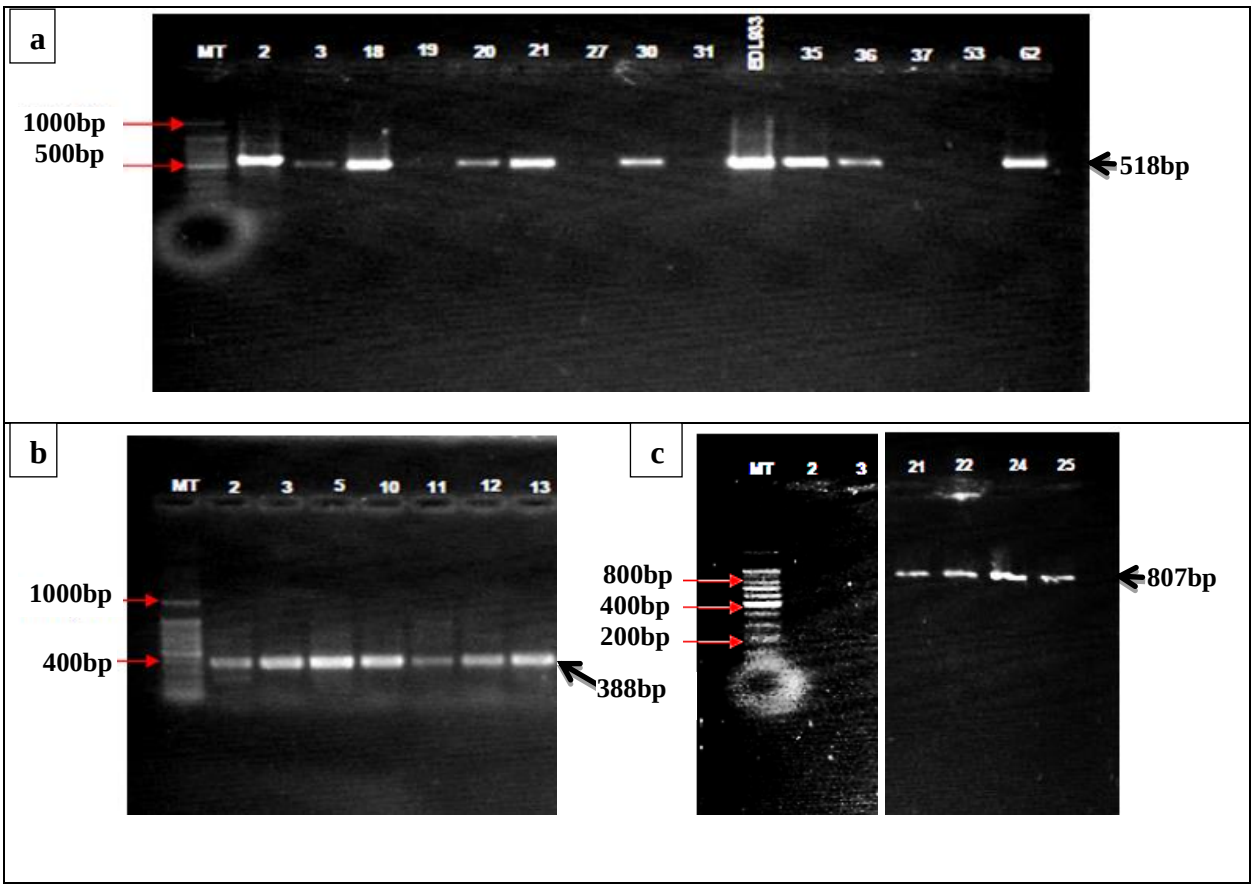

Fig. 2. Electrophoretic analysis of representative PCR products. PCR products (15 µL) from each amplified sample, together with that of the positive control (EDL933), were loaded in wells of 1.5% agarose gel in parallel with a 100 bp molecular weight marker DNA (MT) and separated by electrophoresis. (a) electrophoretic profiles showing the 518 bp expected for the common sequence of *stx*1 and *stx*2 genes, (b) the 388 bp for *stx*1 gene, and (c) the 807 bp for *stx*2

Table 2. Frequency of *stx*1/*stx*2 gene common sequence and *stx*1 and *stx*2 gene sequences separately, found in positive animals

	Total of animals	Presence		Absence	
		(n)	(%)	(n)	(%)
Common sequence for *stx*1 and *stx*2 genes	(n = 200)	61	30.5	139	69.5
*stx*1 gene in positives	(n = 61)	61	100	00	00
*stx*1 gene alone in positives	(n = 61)	51	83.6	10	16.4
*stx*2 gene in positives	(n = 61)	10	16.4	51	83.6
*stx*2 gene alone in positives	(n = 61)	00	00	61	100

Table 3. Distribution of *stx*1 and *stx*2 genes in carrier animals and herds

		Genes		
		*stx*1 only	*stx*1 and *stx*2	*stx*2 only
Cows	(n)	51	10	0
	(%)	83.6	16.4	0
Herds	(n)	14	4	0
	(%)	77.8	22.2	0

Discussion

In the present study our main aim was to evaluate the presence of STEC in Algerian herds raised in districts of the Blida governorate. A sensitive PCR method was used to detect the presence of specific STEC sequences in the bacterial genome in faecal samples from each herd. The results clearly showed the presence of infected animals with a carrier rate reaching 18 out of the 27 investigated herds. However, the carriage rate was found to be variable (4.2% to 100%) in the 18 positive farms. These data are similar

to those found in the north-western part of Spain where the frequency of positive animals in a herd was found to range from 0% to 100% (5).

The faecal excretions with STEC, expressed either on an individual scale (30.5%) or by herd (66.7%), may vary for various reasons. The prevalence of STEC by farm can be assessed more accurately when at least two samples of faeces rather than a single one are used. In fact, several authors have shown that a single negative sample is inconclusive for the animal not being a shedder or a potential one (25). The intermittent or even *ad hoc* nature of the excretion and the seasonal effect were previously highlighted (17, 30). In addition, the existence of so-called super-shedding animals seems to be related to the variations in the rate of faecal excretion of STEC and thus contributes to the strains' release into the environment and their transmission between individuals of the same herd (80% of cases) (20).

It is well known and established now that ruminants are a reservoir for both O157 and non-O157 STEC and the carriage is usually asymptomatic. This healthy status of animals carrying pathogens–which induce illness in humans makes the diagnosis of the carrier cattle difficult because they present no symptoms. Although the presence of STEC has been reported worldwide in cattle, inducing outbreaks mainly in industrialised and emerging countries (USA, Canada, Brazil, Australia, Japan, Europe, and India), there are discrepancies in the rates of identification across the related studies. The observed differences vary according to the selection of farms, animals, frequencies, methods of sampling, and diagnosis.

The national average prevalence of asymptomatic carriage of STEC were found to be greatly variable worldwide. For example, in Europe the proportion of STEC-positive cattle was found to be as low as 2.8% in the UK (32) and as high as 75% in Norway (29). In France, the prevalence of STEC has been estimated as high as 70% from the faecal samples of cattle in the area of Clermont Ferrand (23), while in Germany this proportion was found to be moderate (24.7%) in dairy cows (21).

Although the screening has focused on faeces, our results differ in the low carriage rate of the *stx*2 gene in comparison with other authors (26), who found that 60% of STEC carrier animals were associated with the *stx*2 gene only and 23% with the *stx*1 + *stx*2 genes in tandem. The high frequency of *stx*2 gene occurrence in adult cows has been reported by few authors (1, 13). The low rate of *stx*2 occurrence might be associated with the relatively young age of animals in Algerian herds. Indeed, 73% of herds are composed of animals below five years of age.

The presence of Stx2 Shiga toxin is more often associated with the risk of developing HUS in humans than the strains producing Stx1 (6, 15). In addition, Siegler *et al*. (27) showed that Stx2 is 1000 times more toxic than Stx1 for the human renal endothelial cells *in vitro* and more involved in the development of HUS in an experimental monkey model of STEC pathogenesis.

The human pathogenic strains of STEC have been isolated from samples of patients with diarrhoea. Although they were transmitted mainly through diet, 30% of the infections are transmitted by man-to-man contact, by animals, or by water (31). The main food vector is meat, but there are also other food products that have been incriminated (22, 28), such as the sprouted fenugreek seeds responsible for the epidemics that took place in 2011 in France and Germany (11). In the northern region of Africa, food contamination by STEC has already been reported in Egypt, Tunisia, and Morocco. In Algeria, a few studies have been conducted and they described the meat contamination by *E. coli* O157:H7 STEC with rates reaching 7.8% (2, 8). This carriage clearly indicates the high potential threat to the consumer's health.

The molecular characterisation of the Shiga toxin genes in faeces of cattle enabled the highlighting of the asymptomatic occurrence of STEC in dairy farms from the Blida governorate. This carriage represents a potential reservoir of infection for humans through contamination of animal products (meat, milk, and other derived products) and the environment (water, pastures). The demonstration of the STEC reservoir in the studied farms pointed out the necessity of systematic monitoring not only of animals, but also the food products. Although the proportion of *stx*2 gene, which is especially associated with increased virulence and exacerbated STEC pathogenicity, is undetectable, the association of the *stx*1 and *stx*2 genes is not to be neglected.

Conflict of Interests Statement: The authors have no conflict of interest regarding the publication of this article.

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