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Original article

ISOLATION AND CHARACTERISATION OF SHIGA TOXINS PRODUCING *ESCHERICHIA COLI* IN DAIRY COWS IN THE GOVERNORATE OF BLIDA (ALGERIA)

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Summary

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The Shiga toxin producing *Escherichia coli* (STEC) are considered to be one of the most important groups of emerging public health pathogens with cattle being the main reservoir. The objective of this study was to isolate and characterise *Escherichia coli* Shiga toxins in dairy cattle farms. A total of 252 faeces samples were collected from healthy cows belonging to 37 farms. PCR screening of samples for the common sequences of *stx1/stx2* genes and *stx1* and *stx2* genes resulted in a STEC faecal excretion prevalence of 59.5% at the farm level and 26.6% at the individual level. Among positive animals, 85.1% carried STEC with a single *stx1* gene and 14.9% with the *stx1* and *stx2* genes. Immunomagnetic separation was performed on 40 PCR-positive samples (10/10 positive for the *stx1* and *stx2* genes and 30/57 positive only for *stx1*). Biochemical identification revealed the presence of 66 *E. coli* strains (27.5%). The search for virulence genes on these strains by PCR showed that only twenty-two (33.33%) were STEC. The presence of the *stx1*, *stx2*, *ehx* and *eae* genes was characterised in 30.3%, 4.54%, 13.63% and 1.51% of the strains, respectively, indicating that the virulotype with *stx* alone was dominant. Serological identification showed the absence of O157 sero-groups and the presence of O1(2), O2, O18(2), O128 sero-groups. The susceptibility testing of STEC showed 68.18% resistance to chloramphenicol, 63.64% to neomycin, 59.1% to ampicillin, 22.73% to trimethoprim + sulfamethoxazole and 9.1% to amoxicillin + clavulanic acid and nalidixic acid. Four strains showed multi-resistance. Bovine carriage of STEC constitutes a public health risk by contamination of milk and meat. To protect human health, it is necessary to limit the bovine STEC shedding.

Key words: Algeria, cattle, faeces, genes, Shiga toxin producing *Escherichia coli*, virulence

INTRODUCTION

Shiga-toxin-producing *Escherichia coli* (STEC) have been implicated in several worldwide epidemics and sporadic cases resulting from the consumption of contaminated animal foodstuff (Werber et *al*., 2007). However, other modes of transmission of these pathogens to humans are increasingly reported (Blackburn et *al*., 2004; Rangel et *al*., 2005). Non-O157 STEC infections are more common than O157:H7 serotype infections and account for 80% of gastroenteritis as indicated by the national surveillance database in Germany (Werber et *al*., 2008). The pathogenicity of STEC is attributed to the *stx1* and *stx2* genes which encode Shiga toxins 1 and 2, the *eae* gene coding for the virulence factor intimin and the *ehx* gene encoding the enterohaemolysin A. The combination of these genes is known to increase the risk and severity of infections (Boerlin *et al.,* 1999). STECs have been isolated from a large number of animal species. However, in cattle their presence and excretion are mostly asymptomatic.

Although data on the presence of STEC in Africa are very limited (Effler et *al*., 2001; Raji et *al*., 2006), *E. coli* O157 or other STECs have been found in patients and foodstuff from more than twenty countries (Magwira et *al*., 2005; Al-Gallas et *al*., 2007; Badri et *al*., 2009). In Algeria, very few studies have been performed to detect the presence of these bacteria in foodstuffs such as meat (Chahed et *al*., 2006; Barka et *al*., 2014). It is therefore essential to identify bovine carriers of these pathogens because they are considered as the natural reservoir.

In this work, the prevalence of Shiga toxin-producing *Escherichia coli* (STEC) in dairy cattle herds from farms located in the governorate of Blida, North Algeria was studied. To this end, evaluation of asymptomatic STEC carriers was determined by PCR on faecal samples. Positive samples from the first screen were further characterised in terms of STEC strains and virulotypes. The sensitivity to antibiotics of these strains was also analysed.

MATERIALS AND METHODS

Samples collection

The present study involved 252 faecal samples collected from the rectum of healthy adult dairy cows belonging to 37 farms in the governorate of Blida, Algeria. Samples were packaged in sterile 100 mL jars and transported under cold conditions to the laboratory of the Institute of Veterinary Sciences (Blida University 1) for processing.

Screening for the stx1 and stx2 genes by PCR on feces

The adopted approach was based on the detection of common sequence of *stx*1/*stx*2 genes as a screening test, subsequently followed by seeking the specific sequences of *stx*1 and *stx*2 genes on positive samples. Sample processing, DNA extraction as well as PCR detection of the common sequence and the *stx1* and *stx2* genes were performed according to the protocol described by Baazize-Ammi et *al*. (2015). 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.

Immunomagnetic separation and biochemical identification of strains

From the PCR positive specimens, 40 samples (10 *stx1* and *stx2* positive samples and 30 samples positive only for *stx1*) were selected for isolation and cha-

racterisation of *E coli* O157. This was done by immunomagnetic separation. Briefly, 25 g of faeces were added to 225 mL of Trypticase-soy broth, homogenised in a Stomacher® 400 Lab Blender (Seward) and incubated at 37 °C for 6 hours for enrichment. The samples thus treated were used for immunomagnetic separation using Dynabeads anti *E. coli* O157 (Invitrogen Dynal, Norway) according to the manufacturer's instructions. The immuno-concentrates obtained were spread on sorbitol MacConkey agar plates and incubated at 37 °C for 24 hours. From the culture plates six characteristic sorbitol-positive colonies and six negative sorbitol colonies were selected and purified for biochemical identification by API 20^{E} strips (BioMérieux, France).

Screening for stx1 and stx2 genes on E.coli strain

Using these *E coli* strains, virulence factors (*stx1*, *stx2*, *eae* and *ehx*) were sought by PCR. DNA extraction was performed with InstaGene ™ Matrix reagent (BIO-RAD) according to manufacturer's instructions. Then, GoTaqFlexi DNA Polymerase (Promega, USA) and oligonucleotide primers (Eurogentec, France) were used (Table 1). The PCR mix was prepared as described by Baazize-Ammi *et al.* (2015). The amplification was carried out in a Mastercycler gradient thermocycler (Eppendorf, Germany) according to the following programme: initial denaturation at 94 °C for 3 min, 35 cycles (each comprising a denaturation at 94 °C for 90 s, hybridisation at 59 °C for 90 s and elongation at 72 °C for 120 s), followed by a final elongation at 72 °C for 5 min (Baazize-Ammi et *al*., 2015).

Serological identification of STEC strains

Serological identification was performed on *E. coli* Shiga toxin strains using the *E. coli* O157 Latex kit (Diagnostic Reagents Oxoid) and Seroscreen DR0300M dry spot kit (Oxoid), according to manufacturer's instructions. The other serogroups were demonstrated using monovalent and polyvalent sera (Eurobio, Sifin) anti somatic O antigens by performing a slide agglutination.

Study of STEC strains sensitivity

For the study of the susceptibility of the STEC strains, a range of eleven antibiotics packaged in the form of discs with a diameter of 9 mm (Oxoid) with the charges recommended by the Algerian Network of the Surveillance of the Resistance of Bacteria to Antibiotics (2011) was used. The antibiogram was performed using the Mueller Hinton agar diffusion method.

Table 1. Nucleotide sequences of primers used

Virulence factors	Primer code	Sequence (5'-3')	bp	References
stx1	B54 B55	AGAGCGATGTTACGGTTTG TTGCCCCCAGAGTGGATG	388	China *et al.* (1996), Carneiro *et al.* (2006)
stx2	B56 B57	TGGGTTTTTCTTCGGTATC GACATTCTGGACTCTCTT	807	
eae	B52 B53	AGGCTTCGTCACAGTTG CCATCGTCACCAGAGGA	570	Fremaux *et al.* (2006)
ehx	hly AF hly AR	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	534	

RESULTS

The detection of the common sequence of *stx1/stx2* genes, based on a PCR screening method, revealed that 67 cows were positive, giving an overall positive rate of 26.6% in individuals and a rate of 59.5% in cattle farms. The separate detection of either *stx1* or *stx2* genes among the positive samples demonstrated that 57 cows carried STECs with the *stx1* gene alone (85.1%) corresponding to 81.8% at the breeding level and 10 cows carrying STECs having simultaneously the *stx1* and *stx2* genes – 14.9% corresponding to 18.2% at the farm level (Table 2).

Biochemical characterisation on API 20^{E} strips of colonies from Sorbitol MacConkey agar plates allowed identification of 66 *E. coli* strains. The PCR analysis of identified strains for the virulence factors *stx1*, *stx2*, *eae* and *ehx* revealed that only 22 strains (33.33%) were STEC positive because of carrying *stx1* and/or *stx2* genes (Fig. 1). Interpretation of the combined results of virulence factors yielded 5 virulotypes, with a predominance of type 1 ($stx1^{+}$ $stx2^{-}$ eae^{-} ehx^{-}), followed by type 2 ($stx1^{+}$ $stx2^{-}$ eae^{-} ehx^{+}) (Table 3).

The serological identification allowed the characterisation of six strains belonging to serogroups O1(2), O2, O18(2),

Table 2. Distribution of *stx1*, *stx1* and *stx2*, and *stx*2 genes amongst individual animals and herds

			Common sequence	*stx*1 gene	*stx*1/*stx*2 genes	*stx*2 gene
Herds	Total (n=37)	(n)	22			
		(%)	59.5			
	Positive (n=22)	(n)		18	4	0
		(%)		81.8	18.2	0
Animals	Total (n=252)	(n)	67			
		(%)	26.6			
	Positive (n=67)	(n)		57	10	0
		(%)		85.1	14.9	0

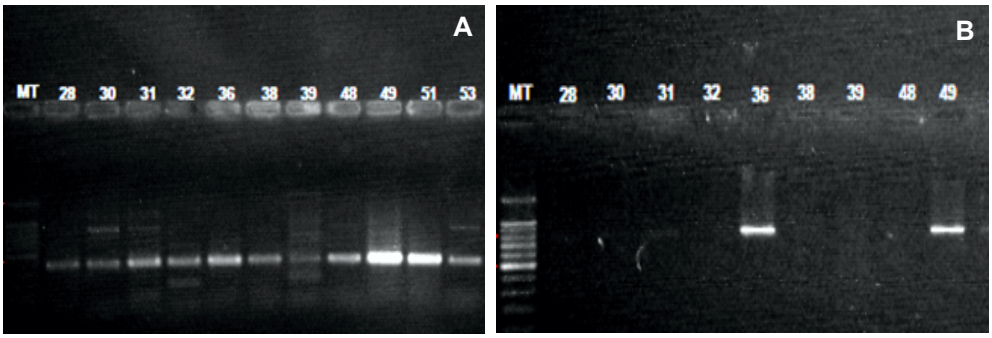

Fig. 1. Electrophoretic analysis of representative PCR products. PCR products (15 µL) from each amplified sample, together with that of the positive control (EDL933), were loaded in wells of 1.5% agarose gel in parallel with a 1000 bp molecular weight DNA marker (MT) and separated by electrophoresis. **A.** Electrophoretic profiles showing the 388 bp product for *stx1* gene (lanes 28-51: *stx*1 positive samples; **B.** the 807 bp product for *stx2* (lanes 36 and 49: *stx*2 positive samples).

O128 as well as 16 other nonagglutinable isolates (Table 3).

The results of the antibiotic susceptibility study of the 22 STEC strains indicated resistance in 68.18% of strains to chloramphenicol, in 63.64% to neomycin, in 59.1% to ampicillin, in 22.73% to trimethoprim with sulfamethoxazole, and in 9.1% amoxicillin+clavulanic acid and nalidixic acid. Furthermore, multiresistance against two and more antibiotics at a time, was detected for four strains (18.18%) (Table 4).

DISCUSSION

The PCR search for the common sequence of *stx1/stx2* genes in 252 faecal samples revealed an individual prevalence rate of 26.6%. From these, 85.1% carried only the *stx1* gene while 14.9% had both *stx1* and *stx2* genes, for an overall rate of 59.5% for herds. The presence of STEC in cattle has been reported in many countries, the data are numerous and vary according to individual studies. The numerous observed differences derive from the choice of farms, breed of animals, sampling frequency and methods, and types

Table 3. Combined results for serogroupes and virulence factors in 22 STEC strains

Serogroups	Virulence factors (by PCR)				Virulotype
	stx1	*stx2*	*eae*	*ehx*	
N.A.	+	–	–	-	1
N.A.	+	–	–	+	2
N.A.	+	–	–	–	1
N.A.	+	–	–	+	2
O:1	+	–	–	+	2
O:18	+	–	–	–	1
N.A.	+	–	–	+	2
N.A.	+	–	–	+	2
N.A.	+	–	–	–	1
N.A.	+	–	–	–	1
O:1	+	–	–	–	1
N.A.	+	–	–	+	2
O:128	+	–	–	+	2
N.A.	+	–	–	–	1
O:18	+	–	–	–	1
N.A.	+	–	–	–	1
O:2	–	+	–	+	5
N.A.	+	+	–	–	3
N.A.	–	+	+	–	4
N.A.	+	–	–	–	1
N.A.	+	–	–	–	1
N.A.	+	–	–	+	2

N.A.: non agglutinable; Virulotypes: ($stx1^{+}stx2^{-}eae^{-}ehx^{-}$)=1; ($stx1^{+}stx2^{-}eae^{-}ehx^{+}$)=2; ($stx1^{+}stx2^{+}eae^{-}ehx^{-}$)=3; ($stx1^{-}stx2^{+}eae^{+}ehx^{-}$)=4 and ($stx1^{-}stx2^{+}eae^{-}ehx^{+}$)=5.

Table 4. Strain distribution according to their susceptibility and resistance

Antibiotics	Strain			
	Susceptibility		Resistance	
	n	%	n	%
Ampicillin	9	40.90	13	59.1
Amoxicillin + clavulanic acid	20	90.90	2	9.1
Ceftiofur	22	100	0	0
Neomycin	8	36.36	14	63.64
Gentamicin	22	100	0	0
Trimethoprim + sulfamethoxazole	17	77.27	5	22.73
Nalidixic acid	20	90.90	2	9.1
Enrofloxacin	22	100	0	0
Colistin	22	100	0	0
Chloramphenicol	7	31.82	15	68.18
Nitrofurantoin	22	100	0	0

of analysis (Islam et *al*., 2008; Rivera et *al*., 2012).

Isolation and characterisation revealed only 22 STEC strains. For samples tested positive for *stx* genes by PCR and for which STEC could not be isolated, different hypotheses can be proposed: the *stx* element detected by PCR could come from free DNA resulting from bacterial lysis, bacteria found in a viable non-cultivable form (Colwell et *al*., 1985), or bacteriophages carrying the *stx* gene (Schmidt et *al*., 2001). This low STEC level could be also explained by the probable loss of *stx* genes occurring during strain isolation. This phenomenon has been reported by Karch et *al*. (1992) for non-O157 EHEC strains and by Feng et *al*. (2001) for EHEC O157 strains.

In this study, predominance of the *stx1* gene, followed by the *ehx* gene was observed, while the *stx2* and *eae* genes occurred at lower levels. These results are in line with the work of Scotland et *al*. (1990) and Willshaw et *al*. (1992) who reported that production of *stx1* toxin alone was much more common in non-O157:H7 *E coli* strains. Similar findings were observed by Tayzar et *al*. (2013), while higher rates of *stx2* gene were reported by Fernandez et *al*. (2010), Bosilevac & Koohmaraie (2011) and Ballem et *al*. (2020). It is important to recall that the *stx2* toxin is more cytotoxic than *stx1* and that *stx2* is associated with high virulence in humans (Rasooly & Do, 2010).

The *eae* gene allowing the attachment and erasure of intestinal cells, fundamental prerequisite for the expression of full virulence of EHEC strains, was carried by one strain only (1.51%). This low rate observed does not necessarily indicate that these strains are not pathogenic for humans. Indeed, atypical strains (absence of the *eae* gene) have already been implicated in haemolytic-uremic syndrome cases in many countries (Werber et *al*., 2007).

Concerning the *ehx* gene, our results revealed a rate of only 13.63%. According to Sandhu et *al*. (1996), enterohaemolysin is produced by 98% of non-O157 *eae* positive *E coli* and by only 36% of *eae* negative strains isolated from cattle, sug-

gesting a high correlation between attachment factor (intimin) and the production of enterohaemolysin (Ehx). Altogether, our data suggest that all isolated STEC strains carry only two genes at most, with a predominance of the *stx* virulotype alone. Indeed, according to Döpfera et *al*. (2012), this virulotype is found at a higher rate than other virulotypes containing another factor (*eae* and *ehx*) in combination with the *stx* gene. According to Thierry et *al*. (2020), a total of 10 different virulence patterns were recovered amongst animal species.

Serotyping of the strains showed the absence of the serogroup O157, which could be explained by the intermittent or punctual nature of the excretion and by a seasonal effect. It should be noted that a high prevalence has been reported during summer (Garber et *al*., 1999). As far as this study was concerned, almost all samples were taken during autumn, winter and spring, from October until April. In dairy farming, the proportion of animals excreting STEC O157 could be estimated at 0.2 to 48.8% of animals (Hussein & Sakuma, 2005). For the other identified serogroups (O1, O2, O18 and O128), they reflect the wide variety of serotypes characterised in cattle, such as 77 different serotypes in dairy cattle faeces and 250 in beef cattle faeces (Hussein & Sakuma, 2005; Hussein & Bollinger, 2005).

The results of the sensitivity study of STEC strains to antibiotics showed resistance to chloramphenicol 68.18%, neomycin 63.64%, ampicillin 59.1%, trimethoprim+sulfamethoxazole 22.73%, amoxicillin+clavulanic acid and nalidixic acid 9.1%. This resistance to antibiotics has been reported by many authors for STEC isolated from various sources (Cergole-Novella et *al*., 2011; Hossain et *al*., 2011; Rehman et *al*., 2013).

CONCLUSION

Based on the results obtained, it may be affirmed that dairy cattle in the governorate of Blida are a reservoir of STEC. It is probable that certain strains isolated during this study are potentially pathogenic for humans or may become so. These strains can be transmitted to humans through direct contact with carrier animals and through raw milk. Waste from these animals can also contaminate the environment and agricultural production through the use of manure.

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