REVISED MANUSCRIPT

Prevalence of Enterohaemorrhagic *Escherichia coli* from serotype O157 and other attaching and effacing *Escherichia coli* on bovine carcasses in Algeria

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Running header: AEEC on bovine carcasses in Algeria

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Summary

Aims. Bovine meat is the principal source of human contamination of attaching and effacing *E. coli*, including Enterohaemorrhagic *E. coli* O157. The aim was to study the prevalence of these strains on bovine carcasses in Algeria.

Methods & Results. Two hundred and thirty carcasses were swabbed and analysed by classical microbiological methods for total *E. coli* counts and for the presence of pathogenic *E. coli*. The *E. coli* counts were high, with a 75^{th} percentile of 444.75 CFUs $(cm^2)^{-1}$. More than 7% of the tested carcasses were positive for *E. coli* O157. Eighteen *E. coli* O157 strains were isolated and typed by multiplex-PCR. The main isolated pathotype (78%) was *eae*+ *stx2*+ *ehxA*+. Other attaching and effacing *E. coli* were also detected from carcasses by colony hybridisation using *eae*, *stx1* and *stx2* probes. Thirty carcasses (13%) harboured at least one positive colony. Sixty-six positive colonies (2.9%) were isolated. The majority (60.6%) of the positive strains harboured an EPEC-like pathotype (*eae*+ *stx*-). Only three EHEC-like (*eae*+ *stx1*+) colonies were isolated from the same carcass. These strains did not belong to classical EHEC serotypes.

Conclusions. In this study, the global hygiene of the slaughterhouse was low, as indicated by the high level of *E. coli* count. The prevalence of both *E. coli* O157 and other attaching and effacing *E. coli* was also high, representing a real hazard for consumers.

Significance & Impact of Study. This is the first study of this type in Algeria, indicating [OR and indicates??] that the general hygiene of the slaughterhouse must be improved.

Key words: AEEC-bovine-carcasses-Algeria

INTRODUCTION

Since the discovery of the Shiga toxin by Kiyoshi Shiga in the late 1800s, a lot of data have been accumulated about Shiga toxin-producing bacteria. However, it was not until the late 1970s that *Shigella dysenteriae* was associated with the clinical conditions known as haemolytic uraemic syndrome (HUS), thrombocytopaenia and haemolytic anaemia (Koster et al. 1978). At this time, it was discovered that certain strains of *Escherichia coli* could produce Shiga toxins (Konowalchuck et al. 1977). These *E. coli* are now called Shiga toxin-producing *E. coli* or STEC (Calderwood et al. 1996; Mainil and Daube 2005).

The STEC associated with diarrhoea, bloody diarrhoea, haemorrhagic colitis and HUS have been named enterohaemorrhagic *E. coli* or EHEC (Riley et al. 1983).

The EHEC virulence factors are mainly (i) the pathogenicity island LEE (for locus of enterocyte effacement) (McDaniel et al. 1995), which is responsible for the production of attaching and effacing lesions (Moon et al. 1983) and diarrhoea and (ii) the Shiga toxins mainly encoded by phages, which are responsible for HUS (Paton and Paton 1998).

Enteropathogenic *E. coli* (EPEC) strains are responsible for human diarrhoea and possess the LEE but not the Shiga toxin (*stx*) genes. The expression "attaching and effacing *E. coli*" (AEEC) designates [OR describes??] *E. coli* causing attaching and effacing lesions, and includes both EHEC and EPEC (China 2000).

The presence of the LEE is very often detected by the amplification of the *eae* gene (Gallien 2003) encoding intimin, a major EHEC virulence factor (Jerse et al. 1990). There are two major classes of

stx toxins, *stx1* and *stx2*. Specific PCR systems have been described for *stx1* and *stx2* gene amplifications (Piérard et al. 1997; Gallien 2003). The main serotype involved in outbreaks worldwide is the serotype O157:H7, with 48 documented outbreaks involving 12647 cases and 50 deaths (Espié and Leclerc 2002). This justifies subjecting EHEC O157 to very careful survey [OR This justifies the need to subject EHEC O157 to very careful survey/scrutiny OR This justifies the fact that EHEC O157 has been subjected to very careful survey/scrutiny??]. It remains the main serotype involved in HUS.

The major source of human EHEC contamination is contaminated and undercooked beef. In a recent study, we observed a prevalence of 0.89% on chilled beef carcasses in Belgium (Chahed et al. 2005).

Nevertheless, many other serotypes (O26, O103, O111, O118, O128, O145…) have been involved in sporadic cases (Gyles et al. 1998). Of these serotypes, some produce enterohaemolysin, which is a plasmid encoded protein (Schmidt et al. 1995). Its role in virulence has not been clearly established, but it is an interesting diagnostic tool. Indeed, enterohaemolysin activity can be visualised on blood agar plates (Beutin et al. 1996). Moreover, the *ehxA* gene encoding enterohaemolysin can also be amplified by PCR (Gallien, 2003).

In a previous study (China et al. 1996) we developed a multiplex conventional PCR for *eae*, *stx1* and *stx2* genes. Nevertheless [OR Moreover??], many conventional multiplex PCR[s??] have been developed for STEC (Franck et al. 1998; Fagan et al. 1999; Gallien 2003; Osek 2003). Moreover, [OR However??] conventional PCR includes a time consuming and hazardous gel electrophoresis step. Therefore, a real time PCR approach could [OR would??] be faster and safer. The aims of this study were (i) to quantify the faecal contamination of carcasses, (ii) to evaluate the prevalence and to characterise [IT WOULD SOUND BETTER IN REVERSE: to characterise and to evaluate

the prevalence of] STEC and EPEC strains on bovine carcasses in Algeria, using both classical and molecular techniques.

MATERIALS AND METHODS

Sampling. Two hundred and thirty carcasses were sampled during a nine month period in a slaughterhouse in Algeria. Five to ten carcasses were sampled one day a week. The day was changed each week in order to sample the whole production week. The sampling was performed by swabbing four 400 cm^2 zones (1. postero-external zone of the thigh; 2. flank, near the medium lane; 3. thorax, near the medium lane; 4. posterior zone of the front legs) using a first swab (sterile cosmetic cotton) wetted with tryptone salt and a second dry swab. The eight swabs from the sampling of a carcass were pooled in the same sterile bag and transferred to the lab. Information regarding the identification number, the age, the sex and the origin of the carcass was registered.

Classical bacteriology

***E. coli* as a faecal contamination indicator.** The sampling swabs were homogenised in 100 ml buffered peptoned water (Oxoid). One ml of the suspension and of the 10^{-1} dilution were plated on Rapid *E. coli* (REC2) medium (Sanofi-Pasteur). The plates were incubated for 24 hours at 44 °C. The purple colonies were counted and the results expressed as CFUS per cm^2. The percentiles were calculated. The percentile of a distribution of values is a number Xp, such that a percentage p of the population values is less than or equal to Xp. For example, the 25^{th} percentile of a variable is a value (Xp), such that 25% (p) of the values of the variable fall below this value.

***E. coli* O157.** The isolation protocol for *E. coli* O157:H7 involved a pre-enrichment at 42 °C in mTSB broth (Oxoid) supplemented with novobiocin (Oxoid) for 6 to 7 hours, followed by an enrichment in MacConkey broth (Oxoid), supplemented with cefixime (0.050 mg l^{-1}) and potassium

tellurite (2.5 mg l^{-1}), and incubated at 37 °C for 18 hours. An immuno-concentration (Dynabeads O157) and a plating on sorbitol-MacConkey agar supplemented with cefixime-tellurite (CT-SMAC, Oxoid) were performed and the plates were incubated for 18 hours at 42 °C, followed by confirmation by latex agglutination (Dryspot *E. coli* O157, Oxoid) and by biochemical gallery (Api20E, Biomérieux). The presence of the H7 antigen was investigated by latex agglutination.

PCR. The reference strains used in this study are listed in Table 1.

The pathotype (*eae, stx1, stx2*) of the EHEC strain was performed [OR obtained??] using the multiplex PCR previously described (China et al., 1996). In addition, *ehxA* primers were added to the multiplex PCR: HlyA460 and HlyA676, generating a 233 bp amplicon (Table 2). The *ehxA* amplicon was purified (ExoSap-IT, Amersham Biosciences) and sequenced (Dynamic ET-terminator Kit, Amersham Biosciences) in order to confirm that the amplicon corresponded to the *ehxA* sequence. The sequencing products were analysed by capillary electrophoresis (Megabace 500, Amersham Biosciences) and the sequences were deduced using Sequence analyser software (Amersham Biosciences). The obtained sequences were aligned with the expected sequences using BlastN software (http://www.ncbi.nlm.nih.gov).

Real time PCR. Genomic DNA was extracted using a commercial kit (Promega Wizard Genomic Kit, Promega). The PCR primers used in this study are listed in Table 1.

New *ehxA* primers (hlyA1814 and hlyA1961) were selected using OLIGO® 6 software (version 6, MedProbe, Norway) in a constant region after the alignment of *ehxA* sequences from EHEC (O157:H7 accession number X86087; O128 accession number AB032930; O111 accession number X94129). The *eae* primers were selected using OLIGO® 6 software in a constant region after the alignment of *eae* sequences from EHEC (*eae* type kappa from O118 strain 6044/95 accession

number AJ308552; *eae* type iota from O145 strain 7476/76 accession number AJ308551; *eae* type alpha from O111:H9 strain 921-B4 accession number AF449417; *eae* type theta from O111:H8 strain CL37 accession number AF449418; *eae* type beta from O26 strain 413/89-1 accession number AJ275113; *eae* type gamma2 from O111:H- strain 95NR1 accession number AF025311) (Zang et al., 2002; Tarr and Whittman, 2002).

For real time PCR, the amplification mixture was: 12.5 µl of IQ™ Sybr® Green supermix (BioRad), 0.25 ul of each primer (40 µM), 2 µl of DNA (50 ng/µl) and 10 µl of PCR grade water. The amplification was performed on an ABI7000 thermocycler (Applied Biosystems). The following cycle was applied (1x 50 °C for 2 min., 1x 94°C for 3 min., 40x [94 °C for 15 sec., 52 °C for 1 min]). Amplification was followed by a melting step (from 60 °C to 95 °C).

Colony hybridisation. After pre-enrichment, 10 µl were plated on SMAC agar and incubated at 42 °C for 18 hours. Ten colonies per sample were selected for the colony hybridisation method (Mainil et al. 1993). The probes (*eae, stx1, stx2*) were obtained and labelled as described previously (China et al. 1998). The plates were duplicated and the positive colonies were tested for the presence of *eae, stx1* and *stx2* genes by PCR (China et al. 1996).

RESULTS

***Escherichia coli* counts.** Two hundred and thirty carcass swabs were analysed for the count of *E. coli* as an indicator of faecal contamination. The results are summarised in Figure 1. The graphic represents the CFUS per cm^2 (log scale) in [OR as a??] function of the percentiles. The results

indicate that 95% of the counts were below 2321.75 CFUs $(cm^2)^{-1}$ and that 75% of the results were below 444.75 CFUs $(cm^2)^{-1}$.

Prevalence of *Escherichia coli* O157. The same 230 carcass swab samples were tested for the presence of *E. coli* O157. Eighteen samples (7.8%) were found to be positive for the presence of *E. coli* O157 by classical microbiological methods. The positive colonies were sorbitol and β-glucuronidase negative. Only two strains were H7 positive (Table 3). The majority of the positive samples were isolated from males (16/18). Moreover, the majority (10/18) of the positive carcasses came from calves (≤ 1 year). Surprisingly, there was no correlation between the *E. coli* count and the presence of *E. coli* O157. Indeed, the samples positive for *E. coli* O157 were not the most *E. coli* contaminated samples.

PCR typing. First a classical multiplex PCR method for *eae, stx1, stx2, ehxA* genes was developed on reference strains (Figure 2). This multiplex PCR was applied to the 18 *E. coli* O157 strains isolated from bovine carcasses. All the tested strains were positive for *eae* and *ehxA* PCR. Moreover, sixteen (88.9%) of these strains were presumptive STEC, since they possessed either *stx1* or *stx2* or both genes. The dominant pathotype was *eae stx2 ehxA* (78%). One strain (5.5%) presented the *eae stx1 ehxA* pathotype and one strain presented the *eae stx1 stx2 ehxA* pathotype. Moreover, two strains (11%) were presumptive EPEC (*eae*+ *stx*- *ehxA*+). Therefore, all the isolated strains were potentially pathogenic for humans.

Real time PCR. In order to improve the molecular typing of STEC strains, a real time PCR method was developed. The idea was to amplify *eae, stx1, stx2* and *ehxA* genes and to differentiate the amplicons using a melting curve discriminating the amplicons by their different melting temperatures (Figure 3). The results obtained for the 18 STEC strains used in this study were the same as those obtained by classical multiplex PCR (data not shown).

Colony hybridisation. In parallel to the research on STEC O157, a strain which is more dangerous for human health, the presence of other STEC strains was investigated by colony hybridisation. The probes tested were *eae, stx1, stx2*. Ten *E. coli* colonies by sample were tested. Thirty carcasses (13%) presented at least one positive colony (Table 4). Interestingly, these positive carcasses were different from those positive for O157 *E. coli*. Sixty-six colonies (3%) were positive for at least one probe. Since 10 *E. coli* colonies were randomly selected, the fact that one colony was positive indicates that this strain represented at least 10% (1/10) of the total *E. coli* population sampled from the [OR a?] carcass.

Of the 66 colonies, 40 presented an EPEC pathotype (*eae*+; *stx*-); 23 presented an STEC pathotype (*eae*- *stx*+) and [OR but??] only three strains presented an EHEC-like pathotype (*eae*+ *stx*+). Interestingly, these three EHEC-like colonies were isolated from the same carcass. None of these three EHEC-like strains agglutinated with the most relevant EHEC non-O157 serotypes (O26, O111, O128, O103, O91) antisera.

DISCUSSION

Faecal contamination on bovine carcasses is the signature of a hygiene problem during the slaughtering process. In 1996 the American Food Safety and Inspection Service (FSIS) proposed microbiological criteria for *E. coli* count on bovine carcasses. The lower limit (m= 80th percentile) was 5 CFUs (cm^2) $^{-1}$ and the upper limit (M=98th percentile) was 100 CFUs (cm^2) $^{-1}$ (Food Safety Inspection Service 1996). Moreover, Belgian legislation is stricter, as the lower limit (m=75th percentile) is 0.7 CFUs (cm^2) $^{-1}$ and the upper limit (M= 95th percentile) is 20 CFUs (cm^2) $^{-1}$, with an acceptable number of intermediary value being 20% (Anonymous 1996). The results obtained in this study indicate that the carcasses were heavily contaminated, as the calculated lower limit (3m=

percentile 75) in this study was 444.75 CFUs (cm^2) $^{-1}$, which is higher than the acceptable upper limits fixed by both US and Belgian legislation. If the Belgian rules were to be applied, 38.1% of the individual *E. coli* counts would be in the unacceptable region, 39.6% (higher than the accepted 20%) would be in the intermediate region and only 22.3% would be in the acceptable region (Figure 1). The situation would be slightly better if the American limits were applied: 46.2% acceptable results, 15.7% unacceptable results and 38.1% intermediate results. Therefore, the first conclusion of this work was [OR is??] that hygiene in the abattoir must be improved and that an HACCP [DO YOU NEED TO GIVE THIS ABBREVIATION IN FULL??] plan should be considered.

The prevalence of *E. coli* O157 on the sampled carcasses was 7.8%. Surprisingly, there was no correlation between the *E. coli* count and the presence of *E. coli* O157. Indeed, the samples positive for *E. coli* O157 were not the most contaminated samples. Nevertheless, the high level of contamination could obscure the main?? [OR real??] issue.

The percentage of O157 *E. coli* positive carcasses found here can be compared to other studies. The percentage is highly variable from one study to another. For example, some percentages have been higher or similar: 17.8% in the USA (Elder et al. 2000), 12% in Italy (Bonardi et al. 2001), 11% (McEvoy et al. 2003) in the Irish Republic and some percentages have been lower: 3.6% (Gun et al. 2003) in Turkey, 1.4% (Chapman et al. 2001) in the UK, 1.02% (Tutenel et al. 2003) [IN WHICH COUNTRY??], 0.89% (Chahed et al. 2005) in Belgium and 1% (Lukasova et al. 2004) in the Czech Republic. Nevertheless [OR However??], these results are sometimes difficult to compare, since the number of samples, the sampled surface and the method of detection were different. Regarding the detection method, an international ISO standard (ISO16654) exists for the detection of *E. coli* O157 (International Standardisation Organisation, 2001). For the sampled surface, a European regulation exists (2001/471/EC, European Commission, 2001) recommending four sampling regions (rump, flank, brisket and neck) of at least 100 cm^2 each. Therefore, it

would?? be useful to follow the standards so that the results can be compared. Moreover, the *E. coli* O157 count has been shown to decrease?? on the carcass during processing (Elder et al., 2000). Therefore, the moment of sampling in the abattoir is also critical.

The major STEC serotype involved in foodborne outbreaks worldwide is the serotype O157:H7. Of the 18 isolated *E. coli* O157 strains in this study, only 2 isolates produced the H7 antigen. Nevertheless, *E. coli* O157nonH7 strains have [also] been shown to be responsible for diseases worldwide (Espié and Leclerc 2002). Indeed, the virulence of these strains has been mainly associated with the production of Shiga toxins encoded by *stx1* or *stx2* genes and with the presence of the pathogenicity island LEE, responsible for attaching and effacing lesions (Paton and Paton 1998; Mainil and Daube, 2005). Moreover, *E. coli* O157:H7 strains possess a virulence plasmid harbouring the *ehxA* gene encoding enterohaemolysin. Of the 18 *E. coli* O157 strains isolated here, all possessed the *eae* LEE-borne gene and were therefore potentially able to produce attaching and effacing lesions. Sixteen (89%) possessed either the *stx1* or the *stx2* genes or both and can therefore be considered as true STEC. The two *stx*-negative strains can be considered as EPEC strains.

In order to determine the presence of a/the?? virulence gene [OR the presence of virulence genes??], many classical PCR systems have been developed in simplex or in multiplex (China et al. 1996; Osek et al. 2003). The most STEC targeted genes have been *eae, stx1, stx2* and *ehxA*. More recently, real time PCR typing methods have been developed in order to avoid the hazardous and time consuming gel electrophoresis step (Ibeweke et al. 2002; Reischl et al. 2002; Nielsen and Andersen 2003). Here, a real time PCR method based on the differences in amplicon melting temperatures was proposed. This method is cheaper in comparison with real time PCR methods based on probes. The major problem was to obtain a PCR efficiency equivalent for each amplification in order to obtain peaks of the same height in the melting curve. This method was

tested in a single [WORD MISSING??] (Figure 1) but multiplexation of the amplifications was also tried with a lack of reproducibility in our case.

Besides O157:H7, other STEC serotypes (O26, O91, O103, O111, O118, O128, O145,…) are involved in sporadic foodborne disease cases. Unfortunately no standard method exists to isolate these strains. Here, a colony hybridisation method was applied using *eae, stx1 and stx2* probes. Around three percent (66/2300) of the tested colonies were positive with at least one probe. The principal pathotype (40 strains=60.6%) was: *eae*+ *stx*-, corresponding to an EPEC pathotype. Interestingly the percentage of EPEC strains ranged from 10 (one positive colony) to 90 percent (9 positive colonies) of the total *E. coli* population on the [OR a??] carcass. Twenty-three strains were only positive for *stx* hybridisation (either *stx1*, or *stx2* or both) but negative for *eae* hybridisation. Finally, 3 colonies presented an EHEC-like pathotype (*eae*+ *stx1*+). These strains were isolated from the same carcasses representing 30% of the *E. coli* population. They did not belong to serotypes O26, O91, O103, O111, O128, O145 or O157 as checked [USE EITHER: as verified OR when checked??] by latex agglutination.

In conclusion, this study indicates that general hygiene must be improved in this Algerian abattoir. Moreover, the relatively high prevalence of AEEC (whether O157 or not) on bovine carcasses indicates that the risk is significant for public health. Therefore, it seems that a surveillance procedure should be established in the country. Finally, clinical data must be collected in order to estimate the real impact of food contamination on human health in Algeria.

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Table 1: Characteristics of PCR primers used

Gene	Primer name and sequence	Size (bp)	OAT* (°C)	Tm† (°C)	Reference
Eae	B52: aggcttcgtcacagttg B53: ccatcgtcaccagagga	570, ‡	51.9	85.0	China et al., 1996
Eae	Eae30: ccggcacaataatgc Eae135: tttgaatccgaacycaattta	126, §	50.4	77.5	This study
Stx1	B54: agagcgatgttacggtttg B55: ttgcccccagagtggatg	388, ‡, §	55.5	85.6	China et al., 1996
Stx2	B56: tgggtttttcttcggtatc B57: gacattctggttgactctctt	807, ‡, §	53.4	87.8	China et al., 1996
EhxA	*HlyA*460: aaacaacgggaaggagag *HlyA*676: acaacatccagccca	233, ‡	53.3	83.3	This study
EhxA	HlyA1814: acgatgtggtttattct HlyA1961: ctccacgtcaccatacatat	167, §	52.2	82.6	This study

*: OAT: optimal annealing temperature as calculated by OLIGO6® software

†: melting temperature of the amplicon as calculated by OLIGO6® software

‡: primers used in classical multiplex PCR

§: primers used in real time PCR

Table 2: Characteristics of reference strains

Name	Type	Serotype	Pathotype	Reference
211	Bovine STEC	Rough	Stx2 ehxA	China et al., 1996
309S89	Bovine STEC	O20	Stx1 stx2 ehxA	China et al., 1996
317S89	Bovine EHEC	Rough	Eae stx1 ehxA	China et al., 1996
EH248	Human EHEC	O103:H2	Eae stx1 ehxA	Szalo et al., 2002
EH298	Human EHEC	O26	Eae stx2 ehxA	Szalo et al., 2002
EH291	Human EHEC	O111	Eae stx1 ehxA	Szalo et al., 2002
EH296	Human EHEC	O26:H11	Eae stx2 ehxA	Szalo et al., 2002
ATCC43888	Human EHEC	O157:H7	Eae ehxA	Hu et al., 1999

Table 3: Identification and origin of bovine carcasses contaminated by *E. coli* O157

Carcass	Serotype	Sex	Age	*E. coli* count (CFUS /cm^2)
1	O157:H7	Male	3 years	$6.9\ 10^{1}$
2	O157:H7	Male	< 1 year	$6.9\ 10^{-1}$
3	O157	Female	> 5 years	$3.4\ 10^{1}$
4	O157	Female	> 5 years	$1.6\ 10^{2}$
5	O157	Male	4 years	$3.4\ 10^{2}$
6	O157	Male	< 1 year	$8.4\ 10^{1}$
7	O157	Male	< 1 year	$1.3\ 10^{2}$
8	O157	Male	< 1 year	$2.4\ 10^{1}$
9	O157	Male	< 1 year	$0.8\ 10^{-1}$
10	O157	Male	< 1 year	$8.9\ 10^{0}$
11	O157	Male	1 year	$1.7\ 10^{0}$
12	O157	Male	< 1 year	$6.5\ 10^{0}$
13	O157	Male	2 years	$1.1\ 10^{2}$
14	O157	Male	2 years	$2.5\ 10^{1}$
15	O157	Male	4 years	$1.3\ 10^{0}$
16	O157	Male	< 1 year	$7.1\ 10^{0}$
17	O157	Male	< 1 year	$8.3\ 10^{1}$
18	O157	Male	4 years	$7.6\ 10^{0}$

Table 4: Pathotypes of *E. coli* detected by colony hybridisation

Carcass	Pathotype of positive colonies
1	1 x eae+ stx1- stx2-
2	1 x eae+ stx1- stx2-
3	4 x eae+ stx1- stx2-
4	1 x eae- stx1+ stx2-
5	1 x eae- stx1- stx2+
6	2 x eae- stx1+ stx2- 1 x eae- stx1- stx2+
7	1 x eae+ stx1- stx2-
8	5 x eae+ stx1- stx2-
9	1 x eae- stx1+ stx2-
10	9 x eae+ stx1- stx2-
11	3 x eae- stx1+ stx2+
12	1 x eae- stx1- stx2+ 8 x eae+ stx1- stx2-
13	1 x eae- stx1+ stx2+ 1 x eae- stx1- stx2+
14	1 x eae- stx1+ stx2 -
15	1 x eae- stx1- stx2+
16	1 x eae+ stx1- stx2-
17	3 x eae+ stx1+ stx2- 1 x eae- stx1+ stx2 -
18	1 x eae- stx1+ stx2-
19	1 x eae+ stx1- stx2-
20	1 x eae- stx1+ stx2 -
21	1 x eae- stx1- stx2+
22	2 x eae+ stx1- stx2-
23	2 x eae- stx1- stx2+
24	2 x eae- stx1- stx2+
25	3 x eae+ stx1- stx2-
26	2 x eae+ stx1- stx2-
27	1 x eae- stx1- stx2+
28	1 x eae- stx1+ stx2+
29	1 x eae+ stx1- stx2-
30	1 x eae+ stx1- stx2-
30/230 (13%)	66 different colonies in 5 pathotypes 40 (60.6%): eae+ stx1- stx2- 10 (15.2%): eae- stx1- stx2+ 9 (13.6%): eae- stx1+ stx2- 4 (6.1%): eae- stx1+ stx2+ 3 (4.5%): eae+ stx1+ stx2-

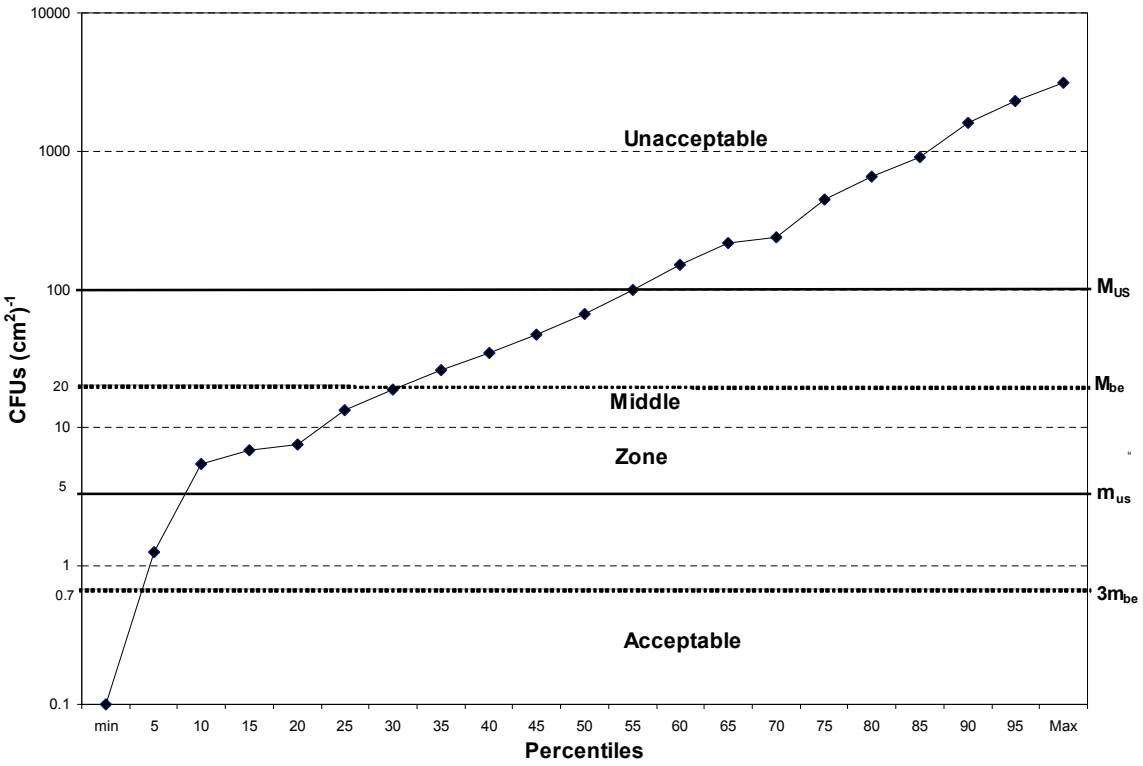

Figure 1: *Escherichia coli* count on bovine carcasses. In CFUs $(cm^2)^{-1}$ in [OR as a??] function of percentiles.

M_{us}: upper limit (100 CFUs $(cm^2)^{-1}$) according to USDA, m_{us} lower limit (5 CFUs $(cm^2)^{-1}$) according to USDA. Mbe: upper limit (20 CFUs $(cm^2)^{-1}$) according to Belgian legislation, $3m_{be}$: lower limit (0.7 CFUs $(cm^2)^{-1}$) according to the Belgian legislation. Three zones were defined: the upper unacceptable zone, the middle zone and the acceptable zone.

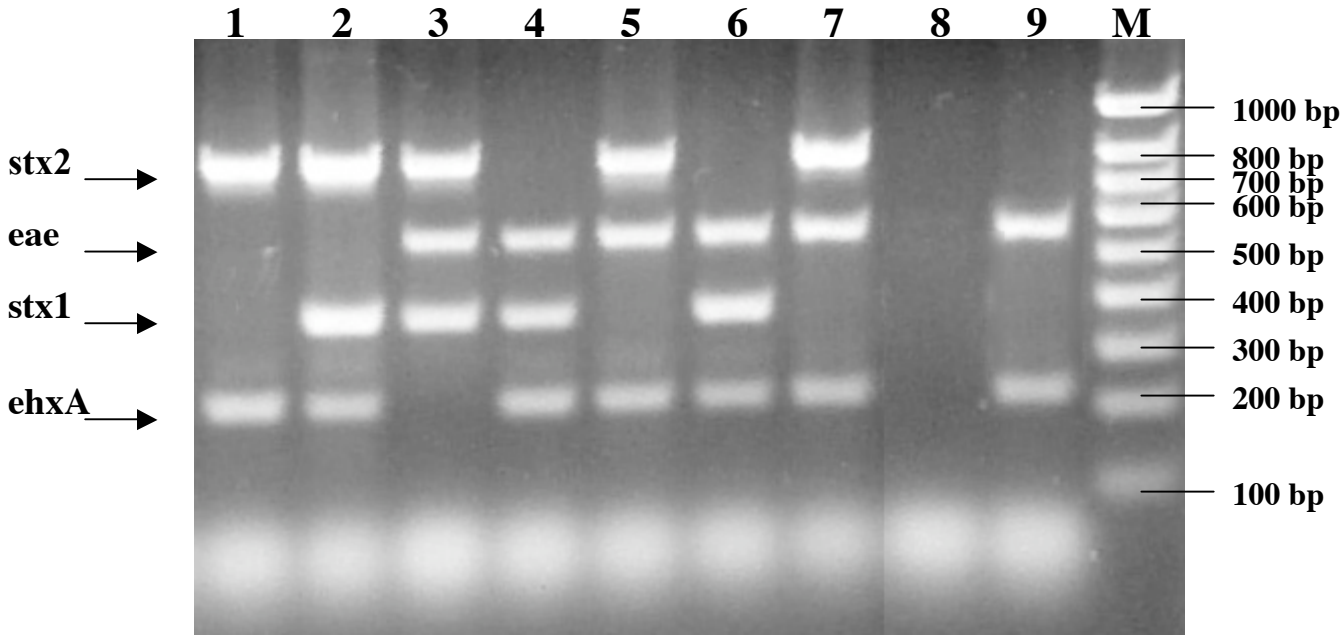

Figure 2: Classical multiplex PCR. After multiplex PCR, amplicons were analysed by agarose gel electrophoresis. 1. Strain 211; 2. Strain 309S89; 3. Strain 317S89; 4. Strain EH248; 5. Strain EH298; 6. Strain EH291; 7. Strain EH296; 8. Non-template control; 9. Strain ATCC43888. M: molecular weight marker; fragment sizes were [OR are??] indicated. Arrows indicate the expected amplicons: *stx2* (807 bp), *eae* (570 bp), *stx1* (388 bp) *and ehxA* (233 bp).

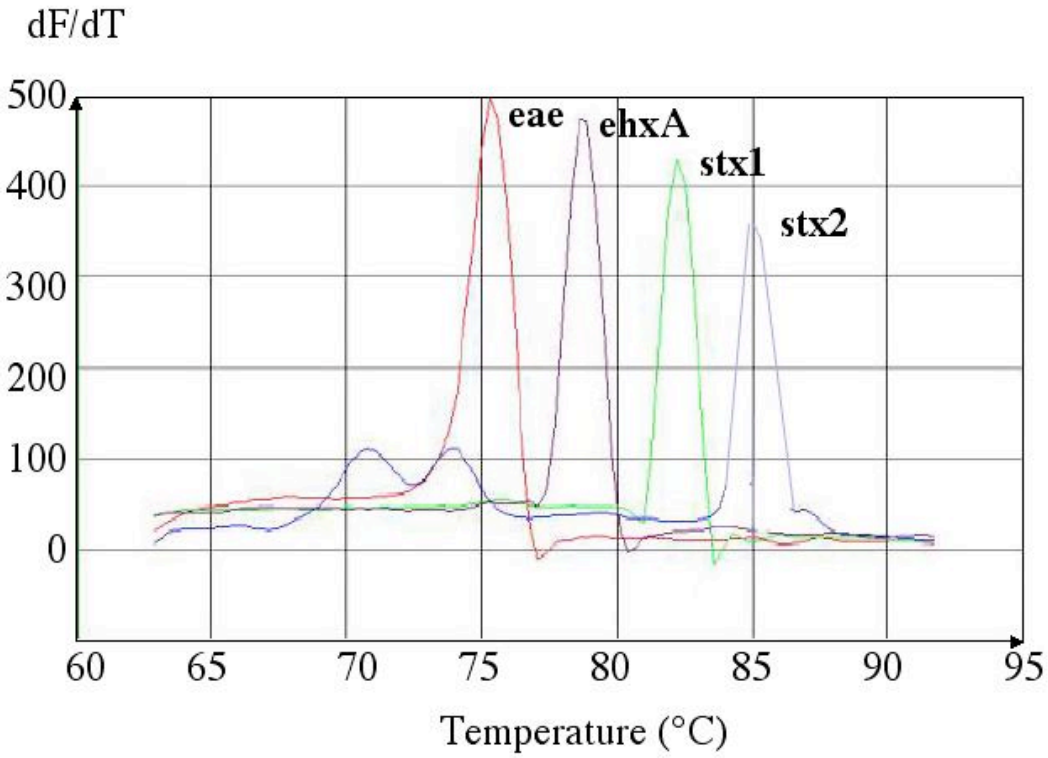

Figure 3: Multiplex real time PCR. Variation of fluorescence (dF) in [OR as a??] function of temperature (°C). Each peak corresponds to a specific amplicon (*stx2*, *stx1*, *ehxA* or *eae*).