JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ANTIMICROBIAL RESISTANCE *ESCHERICHIA COLI* ISOLATED FROM CALVES

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doi: 10.15414/jmbfs.2018.7.4.412-415

ARTICLE INFO

Received 9. 1. 2018
Revised 23. 1. 2018
Accepted 28. 1. 2018
Published 1. 2. 2018

Regular article

OPEN ACCESS

ABSTRACT

A total of 160 *Escherichia coli* strains isolated from calves during one year period were investigated for verotoxigenicity, integron 1 and antibiotic resistance. Selected 30 verotoxigenic or integron 1 positive *E. coli* isolates were studied for antibiotic resistance genes, including the detection of extended-spectrum ß-lactamases and plasmid replicon profiling. Resistances to ampicillin, streptomycin and tetracycline were the most frequent detected and followed by resistance to neomycin, cotrimoxazol, chloramphenicol, florfenicol and enrofloxacin. Two ceftiofur resistant strains were positive for CTX-M 1 with plasmid of FIB incompatibility group. B/O and F1B plasmids were the most frequently carried replicons in VTEC. Majority of strains belonged to commensal phylogenetic group A. In conclusion, commensal *Escherichia coli* of calves are a reservoir of ESBL.

Keywords: VTEC, CTX-M-1, plasmid replicon, *Escherichia coli*, calves

INTRODUCTION

Ruminants, especially cattle, are the main reservoirs of VTEC. Human infection, caused by enterohemorhagic *Escherichia coli* (EHEC), is typically acquired through the ingestion of contaminated food (ground beef, raw milk, meat and dairy products, vegetables, unpasteurized fruit juices) or water **(Griffin and Tauxe, 1991),** through direct contact with animals, or via person-to-person transmission. The prevalences of EHEC O157 were only 0.17%, while for VTEC were 68% of one thousand calf fecal samples **(Cristancho *et al*., 2008).** VTEC O157 prevalence in adult sheep randomly selected at abattoir was 7.1% **(Franco *et al*., 2009).** Recently, a series of reports have described *Escherichia coli* strains carrying CTX-M-type extended spectrum β-lactamases (ESBL) isolated from cattle in some European countries **(Brinas *et al*., 2005, Liebana *et al*., 2006, Meunier *et al*., 2006)**.

The aim of this study was to investigate the verotoxigenicity, integron 1 and antibiotic resistance, including ESBLs in *Escherichia coli* isolated from calves of various farms during one year.

MATERIAL AND METHODS

Isolation and identification of *Escherichia coli*

The transport rectal swabs from calves of various farms were resuscitated in buffered peptone water (Oxoid, Basingstoke, United Kingdom) and than were subcultivated on MacConkey agar (Oxoid, Basingstoke, United Kingdom). The identification of *Escherichia coli* was confirmed using a Triple sugar agar (Imuna, S. Michalany, SK) or Uriselect agar (Bio-Rad Lab., Hercules, California, USA) and Entero test 24 (Erba-Lachema, Brno, CR). A total of 160 *Escherichia coli* were isolated during one year.

Susceptibility test

Minimal inhibitory concentration (MIC) was determined by colourimetric broth microdilution method according to CLSI guidelines (Vet 01-S2 and M100-22) with using antimicrobial agents ampicillin, ampicillin+sulbactam, ceftriaxone, ceftiofur, cefquinome, ceftazidime, ceftazidime+clavulanic acid, streptomycin, spectinomycin, neomycin, enrofloxacin, chloramphenicol, florfenicol, tetracycline and cotrimoxazol. CTX-M production was phenotypically detected by interpretative reading of MIC cephalosporins **(Pitout et al., 2004).**

PCR amplification

Genes for *vt1*, *vt2* and *eaeA* were detected by real time PCR **(Nielsen and Andersen, 2003)** with modification according **Bujnakova *et al*. (2007)**. Integrase 1 (Mazel *et al*., 2000) and integron related genes *aadA* **(Clark *et al*., 1999)**, *dfrA, B* **(Navia *et al*., 2003)**, *sul1* and *sul2* according **Kerrn *et al*. (2002**), *sul3* **(Perreten and Berlin, 2003)**.

Investigation of phenotype positive isolates for antibiotic resistance genes (Table 1) was carried out by PCR amplification of *tetA* and *tet B* **(Guillaume *et al*., 2000)**, bla_{TEM} **(Yates *et al*., 2004)** with DNA sequencing of PCR product, chloramphenicol resistance genes (*cml, cat*) and florfenicol (*flo*) according **Guerra *et al*. (2001)**. After PCR screening for CTX-M groups 1, 2 and **9 (Woodford *et al*., 2006)**, CTX-M1 was confirmed by sequencing using CTX-M-1 group primers **(Carattoli *et al*., 2008)**. The list of used primers is in Table 1. *Escherichia coli* isolates were assigned to phylogenetic groups A, B1, B2 or D according **Clermont *et al*. (2000)** and plasmid replicon typing **Carattoli *et al*. (2005).**

PCRs were carried out in a total volume of 25 µl containing 1 µl of template DNA, each of the primers at 20 pmol, the four deoxynucleoside triphosphates each at 200 µM, PCR buffer, 1.5 mM $MgCl_2$ and 1 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California, USA). PCR amplifications consisted of denaturation at 94 °C for 5 min, 30 cycles at 94 °C for 1 min, at the annealing temperature specific for each primer for 1 min, and at 72 °C for 1 min in a thermal cycler (C1000, Bio-Rad Lab). The amplified DNA was visualized in 1 % agarose gels stained with Gold View Nucleic Acid Stain and a 100 bp ladder was used as standard.

RESULTS

Antibiotic resistance in *Escherichia coli* isolates

The percentages of antibiotic resistance in the 160 *Escherichia coli* isolates recovered from calves during one year are shown in Table 2. Resistances to ampicillin, streptomycin and tetracycline were detected in similar very high percentage of isolates (78.6%/77.5%/76.9%). Resistance to neomycin was detected in 51.5 % isolates; to chloramphenicol and florfenicol in 30% vs. 10% isolates; to cotrimoxazol in 29.3% isolates; to enrofloxacin in 16.8 % isolates; to ampicillin with sulbactam in 8.1% isolates; to ceftiofur (3rd generation) in 4.4 % and cefquinome (4th generation) only in 1.8 % isolates. MIC 90 of enrofloxacin in *Escherichia coli* resistant isolates were very high and reached 32 mg/L.

Table 1 An overview of target genes, primer and probes sequences, product sizes and annealing

Target gene	Sequence (5' -3')	PCR product size (bp)	Annealing temperature
vtx1	GGATAATTTGTTTGCAGTTGATGTC CAAATCCTGTCACATATAAATTATTTCGT FAM-CCGTAGATTATTAAACCGCCCTTCCTCTGGA-BHQ	probe	63
vtx2	GGG CAGTTATTTTGCTGTGGA GAAAGTATTTGTTGCCGTATTAACGA JOE-ATGTCTATCAGGCGCGTTTTGACCATCTT-BHQ	probe	63
eaeA	CATTGATCAGGATTTTTCTGGTGATA CTCATGCGGAAATAGCCGTTA FAM- ATAGTCTCGCCAGTATTCGCCACCAATACC-BHQ	probe	63
intI	GGG TCA AGG ATC TGG ATT TCG ACA TGC GTG TAA ATC ATC GTC G	483	62
sul1	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433	69
sul 2	GCGCTCAAGGCAGATGGCATT GCGTTTGATACCGGCACCCGT	293	69
sul3	GAGCAAGATTTTTGGAATCG CATCTGCAGCTAACCTAGGGCTTTGGA	770	51
dfrA	GTGAAACTATCACTAATGG TTAACCCTTTTGCCAGATTT	474	55
dfrB	GATCGCCTGCGCAAGAAATC AAGCGCAGCCACAGGATAAAT	141	60
aadA	TGA TTT GCT GGT TAC GGT GAC CGC TAT GTT CTC TTG CTT TTG	284	60
tetA	GGCCTCAATTTCCTGACG AAGCAGGATGTAGCCTGTGC	372	55
tetB	GAGACGCAATCGAATTCGG TTTAGTGGCTATTCTTCCTGCC	228	55
cmlA	TGTCATTTACGGCATACTCG ATCAGGCATCCCATTCCCAT	435	55
cat	CCTGCCACTCATCGCAGT CCACCGTTGATATATCCC	623	60
floR	CACGTTGAGCCTCTATAT GG ATGCAGAAGTAGAACGCGAC	868	55
blaTEM	ATGAGTATTCAACATTTCCG CCAATGCTTAATCAGTGAGG	858	55
CTX-M1group	AAAAATCACTGCGCCAGTTC AGCTTATTCATCGCCACGTT	415	52
CTX-M 2group	CGACGCTACCCCTGCTATT CCAGCGTCAGATTTTTCAGG	552	52
CTX-M 9group	CAAAGAGAGTGCAACGGATG ATTGGAAAGCGTTCATCACC	205	52

Two ceftiofur resistant *Escherichia coli* isolates showed MIC for ceftriaxone 32 mg/L and for ceftazidime only 0.25 mg//L, a phenotype indicating for CTX-M.

Mechanisms of antibiotic resistance

A total of 160 *Escherichia coli* strains isolated from calves were selected for verotoxigenicity (*vt1*, *vt2*) and integron 1 (*int1*) by PCR amplification. Thirty verotoxigenic or integron 1 positive *E. coli* isolates were found and studied for antibiotic resistance genes, plasmid replicon profiling and phylogenetic analysis.
Table 3 shows the distribution of antibiotic resistance genotypes within four groups of strains; first are *vt1* and *int1* positive isolates, second *vt2* and *int1* positives, third are VTEC without integron and fourth group of strains are non VTEC with *int1* gene.
The integron 1 associated genes, e.g. the sulfonamide resistance genes (*sul1-3*), encoding dihydropteroate synthases were present in 13 VTEC and 8 nonVTEC strains. However *dfrA* gene encoding trimethoprim resistance gene was present only in two *int1* positive VTEC isolates. The *aadA* gene, which encodes an aminoglycoside adenylyltransferase that confers resistance to streptomycin and spectinomycin, was detected in 11 strains with *int1* (2 nonVTEC and 9 VTEC), however *aadA* was present in 3 VTEC isolates without integron 1, also.
The presence of *tetA* and *tetB* genes, the efflux-type tetracycline resistance genes were detected in all 30 isolates; *tetA* alone in 11 isolates, while *tetB* in 10 isolates; both genes were in 9 isolates. Genes *floR* encoding florfenicol resistance and *cat* gene encoding chloramphenicol acetyltransferase were present in 3 integron 1 positive VTEC isolates and in one nonVTEC one. Gene *floR* and gene *cml* encoding chloramphenicol resistance were detected alone in *int1* positive VTEC, also.
Only simple betalactamase bla_{TEM-1} was identified in 29 ampicillin-resistant isolates. The *blaCTX-M-1* gene (Genbank EU401703) was found in two ceftiofur resistant isolates, which also contained *blaTEM-1*, *tetA* and *vt1*. By PCR-based replicon typing a plasmid of FIB incompatibility group was detected in both isolates. Majority of VTEC strains belonged to commensal phylogenetic group A (83%) and two strains to B1 group. Two VTEC strains of belonged to the pathogenic group B2, contained *eaeA* gene, also. Analysis of ß-lactamase, tetracycline-resistant, florfenicol-resistant, chloramphenicol-resistant and integron 1 genes resulted in the identification of 20 resistance genotypes (Table 3). B/O and F1B plasmids were the most frequently carried replicons in our collection.

Table 2 Antibiotic resistance and MIC90 in 160 *Escherichia coli* recovered from calves

Antibiotic	Resistance %	MIC90 (mg/L)
ampicillin	78.6	128
ampicillin+sulbact.	8.1	16
ceftiofur	4.4	4
cefquinome	1.8	0.25
streptomycin	77.5	256
spectinomycin	25.5	512
neomycin	51.5	256
enrofloxacin	16.8	32
tetracycline	76,9	32
chloramphenicol	30.0	64
florfenicol	10.0	16
cotrimoxazol	29.3	128

DISCUSSION

Antimicrobial agents of the sulphonamide group have been widely used in the treatment of food animal infections, a practice which has been argued to contribute to the maintenance of resistence. The sulphonamide resistance genes *sul l-3*, *dfr* genes and *aadA* gene associated with class 1 integron were found in numerous animal and human bacteria **(Kadlec *et al*., 2005**, **Frank *et al*., 2007, Antunes *et al*., 2007**). Our results are similar to **Zhao *et al*., (2001)**, who first described the presence of integron 1 and antibiotic resistance gene cassettes in VTEC.

Betalactamases are an emerging problem. Third and fourth-generation cephalosporins e.g. ceftiofur and cefquinome, used in animal therapy could select CTX-M-producing *Escherichia coli* **(Cavaco *et al*., 2008)**.

Their use in animals should be carefully considered in view of the critical importance of cephalosporins for humans and the zoonotic potential of ESBL-producing *Escherichia coli*. Moreover wild animals especially rooks and mosquitos (Culex pipiens) living near humans and animals are potential reservoirs of extended spectrum betalactamase producers, also (**Kmet *et al*., 2013, Hleba *et al*., 2016**) .

Although CTX-M enzymes can be carried by various replicons, most (36%) in human *Escherichia coli* were carried by F1A, F1B and FII replicons (Marcade *et al*., 2009). Our two *Escherichia coli* CTX-M1 carried F1B replicon. Plasmid replicon typing is important marker for epidemiological investigation and transposition immunity seems to play an important role in the resistance plasmid diffusion process. **Girardeau *et al*., (2005)** showed that 70% of bovine STEC strains segregated mainly in phylogenetic commensal group B1. In similar way, our collection contained 83% commensal VTEC strains.

Table 3 Genotypic characteristics of 30 *Escherichia coli* isolates

Resistance genotypes	Plasmid profiling	Number of strains
vt1, int1, aadA,sul1,sul2 ,tetA, tetB, bla$_{TEM}$	B/O, FIC, FIB, Frep	3
vt2, int1, tetA, bla$_{TEM}$	F1B	1
vt2, int1, sul2, tetA, bla$_{TEM}$	B/O, F1A, F1B	2
vt2, int1, sul2, tetB, bla$_{TEM}$	B/O, F1C, F1B	1
vt2, int1 ,sul2, tetA, bla$_{TEM}$, *floR*	B/O, F1B	1
vt2, int1,aadA, sul1,sul2, tetA, bla$_{TEM}$	B/O, F1C, F1B	1
vt2, int1,aadA ,sul1,sul2, tetA, tetB	B/O, F1C	1
vt2, int1,sul1, sul2, tetA, tetB, bla$_{TEM}$, *eaeA*	B/O, F1A, F1B, I1	1
vt2, int1,aadA,sul3, tetA, bla$_{TEM}$, *cmlA*	F1B, I1	2
vt2, int1,sul1 ,sul2, tetB, bla$_{TEM}$, *floR ,cat*	B/O, F1B, I1	1
vt2, int ,aadA, dfrA,sul1,sul2, tetA, bla$_{TEM}$ *,floR,cat F1B, I1*		2
vt1, CTX-M1, bla$_{TEM}$, *tetA,*	F1B	2
vt1,vt2, sul2, tetA, bla$_{TEM}$, *eaeA*	B/O, F1B, I1	1
vt1,vt2, aadA,sul1,sul2, tetB, bla$_{TEM}$	F1A, F1B	1
vt1,vt2, aadA, dfrA, sul2, tetA, bla$_{TEM}$	B/O, F1B	1
vt1,vt2, aadA,sul1,sul2, tetA,tetB, bla$_{TEM}$	B/O, F1B, I1	1
int1, dfrA,sul2,tetB, bla$_{TEM}$	B/O, F1A, F1B	3
int1, sul1,sul2, tetB, bla$_{TEM}$	B/O, F1C, F1B	3
int1, aadA,sul1,sul2, tetB, bla$_{TEM}$	nt	1
int1, aadA,sul1,sul,2 tetA, tetB, bla$_{TEM}$, *floR , cat*	nt	1

nt-not typable , all betalactamases (bla) were bla$_{TEM-1}$

CONCLUSION

With this study we have confirmed the presence of antibiotic resistant verotoxigenic *E.coli* in healthy calves entering the food chain. Ceftiofur resistant strains of *E.coli* were positive for CTX-M 1 with plasmid of FIB incompatibility group. We have also shown a broad diversity of genes encoding for antibiotic resistance. Majority of verotoxigenic strains belonged to commensal phylogenetic group A.

Acknowledgements: This study was supported by Slovak grants APVV-16-0171, APVV-14-0274 and VEGA 2/0085/18

REFERENCES

Antunes, P., Machado, J. & Deixe, L. (2007). Dissemination of *sul3*-containing elements linked to class 1 integrons with an unusual 3'conserved sequence region among *Salmonella* isolates. *Antimicrob. Agents Chemother*. 51(4), 1245-1548. https://doi.org/10.1128/aac.01275-06

Brinas, L., Moreno, M.A., Teshager ,T., Y. Sáenz, Y., Porrero, M.C., Domínguez, L. & Torres, C. (2005). Monitoring and characterization of extended-spectrum ß-lactamases in *Escherichia coli* strains from healthy and sick animals in Spain in 2003. *Antimicrob. Agents Chemother*. 49 (3), 1262-1264. https://doi.org/10.1128/aac.49.3.1262-1264.2005

Bujnakova, D., Kmet ,V., Kaclikova, E. & Kmetova, M. (2007). Detection of Shiga toxin-producing *Escherichia coli* in meat swabs by TaqMan real-time PCR targeting *stx* genes. *J. Food. Nutr. Res*. 46(3), 97-100.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins,K.L. & Threlfall, E.L.. (2005). Identification of plasmids by PCR-based replicon typing. *J. Microbiol. Meth*. 63(3), 219–228. https://doi.org/10.1016/j.mimet.2005.03.018

Carattoli, A., Garcia-Fernandez, A., Varesi, P., Fortini, D., Gerardi, S., Penni, A., Mancini, C. & Giordano, A.. (2008). Molecular epidemiology of *Escherichia coli* producing extended-spectrum ß-Lactamases isolated in Rome, Italy. *J.Clin. Microbiol*. 46 (1), 103–108. https://doi.org/10.1128/jcm.01542-07

Cavaco, L. M., Abatih, E., Aarestrup, F.M., & Guardabassi, L. (2008). Selection and persistence of CTX-M-producing *Escherichia coli* in the intestinal flora of pigs treated with amoxicillin, ceftiofur, or cefquinome. *Antimicrob. Agents. Chemother*. 52(10), 3612–3616. https://doi.org/10.1128/aac.00354-08

Clark, N.C., O. Olsvik, J.M. Swenson, C.A. Spiegil & F.C. Tenover. (1999). Detection of a streptomycin/spectinomycin adenyltransferase gene (*aadA*) in *Enterococcus faecalis*. *Antimicrob Agents Chemother* 43, 157–160.

Clermont, O., Bonacorsi,S. & Bingen,E. . (2000). Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl. Environ. Microbiol*. 66(10), 4555–4558. https://doi.org/10.1128/aem.66.10.4555-4558.2000

Cristancho, L., Johnson, R.P., McEwen, S.A. & Gyles, C.L. (2008). *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* in white veal calves *Vet. Microbiol*. 126 (1-3), 200–209. https://doi.org/10.1016/j.vetmic.2007.06.012

Franco, A., Lovari, S., Cordaro, G., Di Matteo, P., Sorbara, L., Iurescia, M., Donati, V., Buccella,C. & Battisti, A.. (2009). Prevalence and concentration of verotoxigenic *Escherichia coli* O157:H7 in adult sheep at slaughter from Italy. *Zoonoses Public Health*. 56, 215–220.

Frank, T., Gautier, V., Talarmin, A., Bercion, R., & Arlet, G. . (2007). Characterization of sulphonamide resistance genes and class 1 integron gene cassettes in *Enterobacteriaceae*, Central African Republic (CAR). *J. Antimicrob. Chemother*. 59, 742–745.

Girardeau, J.P., Dalmasso, A., Bertin, Y., Ducrot, C., Bord, S., Livrelli, V., Vernozy-Rozand, C., & Martin,C. (2005). Association of virulence genotype with phylogenetic background in comparison to different seropathotypes of Shiga toxin-producing *Escherichia coli* isolates. *J. Clin. Microbiol*. 43 (12), 6098-6107. https://doi.org/10.1128/jcm.43.12.6098-6107.2005

Griffin, P. M. & Tauxe,R.V.. (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiol. Rev*. 13, 60–98.

Guerra, B., S., Soto, S.M., Arguelles, J.M., & Mendoza, M.C. (2001). Multidrug resistance is mediated by large plasmids carrying a class 1 integron in the emergent *Salmonella enterica* serotype [4,5,12:i:2]. *Antimicrob. Agents Chemother*. 45(4), 1305–1308. https://doi.org/10.1128/aac.45.4.1305-1308.2001

Guillaume, G., Verbrugge, D., Chasseur-Libotte, M., Moens,W. & Collard, J.. (2000). PCR typing of tetracycline resistance determinants (Tet A-E) in *Salmonella enterica* serotype Hadar and in the microbial community of activated sludges from hospital and urban wastewater treatment facilities in Belgium. *FEMS Microbiol. Ecol*. 32, 77-85.

Hleba L., Kmeť, V., Tóth, T. & Kačániová, M. (2017). Resistance in bacteria and indirect beta-lactamase detection in *E.coli* isolated from culex pipiens detected by matrix-assisted laser desorption ionization time of flight mass spektrometry. *J. Environ. Science and Health - Part B Pesticides, Food Contam., Agric. Wastes*, 52(1), 64-69. https://doi.org/10.1080/03601234.2016.1229466

Kadlec, K., Kehrenberg, C. & Schwarz, S. (2005). Molecular basis of resistance to trimethoprim, chloramphenicol and sulphonamides in *Bordetella bronchiseptica*. *J. Antimicrob. Chemother*. 56(3), 485–90. https://doi.org/10.1093/jac/dki262

Kerrn, M.B., Klemmensen, T., Frimodt-Møller, N. & Espersen, F.. (2002). Susceptibility of Danish *Escherichia coli* strains isolated from urinary tract infections and bacteraemia, and distribution of *sul* genes conferring sulphonamide resistance *J. Antimicrob. Chemother*. 50(4), 513–516. https://doi.org/10.1093/jac/dkf164

Kmet, V., Drugdova, Z., Kmetova, M. & Stanko, M. (2013) Virulence and antibiotic resistance of *Escherichia coli* isolated from rooks. *Annals of Agric Environ. Med.* 20 , 273-275.

Liebana, E., Batchelor, M., Hopkins, K.L., Clifton-Hadley, F.A., Teale, C.J., Foster, A., Barker, L., Threlfall, E. & Davies, R.H.. (2006). Longitudinal farm study of extended-spectrum β-lactamase-mediated resistance. *J. Clin. Microbiol.* 44(5), 1630–1634. https://doi.org/10.1128/jcm.44.5.1630-1634.2006

Marcade, G., Deschamps, C., Boyd, A., Gautier, V., Picard, B., Branger, C., Denamur E., & Arlet, G.. (2009). Replicon typing of plasmids in *Escherichia coli* producing extended-spectrum ß-lactamases. *J. Antimicrob. Chemother*. 63, 67–71.

Mazel, D., Dychingo, B., Webb, V.A., & Davies, J. (2000). Antibiotic resistance in the ECOR collection: Integrons and identification of a novel *aad* gene. *Antimicrob. Agents Chemother*. 44(6), 1568–1574. https://doi.org/10.1128/aac.44.6.1568-1574.2000

Meunier, D., Jouy, E., Lazizzera, C., Kobisch, M. & Madec, J.Y.. (2006). CTX-M-1 and CTX-M-15 type β-lactamases in clinical *Escherichia coli* isolates recovered from food-producing animals in France. *J. Antimicrob. Agents* 28, 402–407.

Navia, M.M., Ruiz, J., Sanchez-Cespedes, J. & Vila, J.. (2003). Detection of dihydrofolate reductase genes by PCR and RFLP. *Diagn. Microbiol. Infect. Dis.* 46(4), 295–298. https://doi.org/10.1016/s0732-8893(03)00062-2

Nielsen, E. M. &Andersen, M.T. (2003). Detection and characterization of verocytotoxin-producing *Escherichia coli* by automated 5' nuclease PCR assay. *J. Clin. Microbiol*. 41(7), 2884-2893. https://doi.org/10.1128/jcm.41.7.2884-2893.2003

Perreten, V. & Boerlin, P. (2003). A new sulfonamide resistance gene (*sul3*) in *Escherichia coli* is widespread in the pig population of Switzerland. *Antimicrob. Agent Chemother*. 47(3), 1169–1172. https://doi.org/10.1128/aac.47.3.1169-1172.2003

Pitout, J.D.D., Hossain, A. & Hanson, N.D. (2004). Phenotypic and molecular detection of CTX-M-ß-lactamases produced by *Escherichia coli* and *Klebsiella spp. J.Clin. Microbiol*. 42(12), 5715-5721. https://doi.org/10.1128/jcm.42.12.5715-5721.2004

Woodford, N.,Fagan, E.J. &Ellington, M.J.. (2006). Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum (ß)-lactamases. *J Antimicrob Chemother*. 57, 154-155.

Zhao S., White, D.G., Ge, B., Ayers, S. , Friedman, S., English, L., Wagner, D., Gaines, S. & Meng,J. (2001). Identification and characterization of integron-mediated antibiotic resistance among shiga toxin-producing *Escherichia coli* isolates. *Appl. Environ. Microbiol*. 67(4), 1558–1564. https://doi.org/10.1128/aem.67.4.1558-1564.2001