

Isolation and characterization Shiga toxin-producing *Escherichia coli* from sheep and goats in Jordan with evidence of multiresistant serotype O157:H7

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ABSTRACT: Ninety-three rectal swabs of lambs and young goats from two extensively and two intensively managed herds in Jordan were taken and examined for Shiga toxin-producing *Escherichia coli* (STEC). The bacteriological examination included the preenrichment of rectal swabs in EC broth with novobiocin, and a subsequent parallel isolation on enterohemolysin agar and immunomagnetic separation with cultivation on CT-SMAC. The STEC O157:H7 strains were demonstrated in 8 of 32 diarrheic lambs 1- to 3-weeks old in one sheep herd with intensive milk production. In the remaining three herds, serogroups O128, O78, O15 and serotype O128:K85 of STEC strains were the most frequent findings. The presence of *stx2*, *ehlyA* and *eaeA* genes in all STEC O157:H7 isolates was confirmed by PCR. In two untypable STEC isolates, *stx2* and *ehlyA* genes were detected. In other STEC non-O157 isolates, only *stx1* and *ehlyA* genes were found. All STEC O157:H7 isolates were resistant against sulphonamides and chloramphenicol, five were also resistant against ampicillin and streptomycin, one against co-trimoxazole. One isolate was resistant against ampicillin, ampicillin-sulbactam, cephalosporins (cefazolin, cefuroxime), monobactams (aztreonam), sulphonamides, co-trimoxazole, aminoglycosides, tetracycline and chloramphenicol. Compared the resistant STEC O157:H7 isolates, the multiresistant isolate had a different RAPD pattern. Of 36 STEC non-O157 isolates, one isolate was resistant against sulphonamides and co-trimoxazole, and another one against ampicillin, streptomycin, sulphonamides and co-trimoxazole. STEC isolates resistant against antimicrobial agents were demonstrated only in herds with intensive management.

Keywords: STEC; EHEC; VTEC; HUS; antimicrobial resistance

The Shiga toxin-producing *Escherichia coli* (STEC), and particularly its O157:H7 serotype, are generally considered the cause of haemorrhagic colitis, and of the hemolytic-uremic syndrome (HUS) and thrombocytopenic purpura in people. These infections occur in all parts of the world including Middle East countries (Lerman et al., 1992; Adwan et al., 2002). Identified human infections were traced to eating undercooked beef (Chapman et al., 1993; Morgan et al., 1993; Bell et al., 1994), drinking contaminated water (Dev et al., 1991; Swerdlow et al., 1992; Danon-Schaffer, 2001), and, sporadically, to a direct contact with infected

animals (Rice et al., 1996; Trevena et al., 1996; Chalmers et al., 1997; Chapman et al., 2000).

Epidemiological studies have shown that the principal sources of human infections are asymptomatic STEC O157 and non-O157 carriers. The frequency of the bacteria shedding in cattle is influenced by various factors (Dargatz et al., 1997; Hancock et al., 1997a,b; Cray et al., 1998). STEC shedding has also been demonstrated in small ruminants (Beutin et al., 1993, 1996a; Kudva et al., 1996; Zschock et al., 2000), and that may represent an equally serious risk for people as cattle infections as evidenced by a recent finding of STEC O157 in sheep milk in

Italy (Rubini et al., 1999) and four cases of HUS recorded in the Czech Republic where the infection vehicle was identified as non-pasteurised goat milk (Bielaszewska et al., 1997).

Just as with other agents of human alimentary infections caused by *Salmonella typhimurium* DT 104 (Threlfall, 2002) and *Campylobacter jejuni* (Swartz, 2002), the STEC strains resistant to antibiotics of the first choice may complicate antimicrobial therapy whose timely application may prevent the development of life-threatening HUS in human STEC infections (Shiomi et al., 1999). Besides having clinical consequences, resistant *E. coli* strains of animal origin may be the source of determinants of resistance for the possible transfer to human strains (Oppegaard et al., 2001).

The aim of the present study was to investigate the STEC prevalence in goats and sheep in Jordan on randomly selected farms with extensive and intensive management, to characterize the strains isolated with regard to their main virulence characters and antibiotic resistance, and, using the results obtained, to estimate possible risks of STEC infections for people resulting from eating sheep and goat products from that territory and contacts with animals.

MATERIAL AND METHODS

Herd characteristics and sample collection.

For the study, two herds of sheep and two herds of goats, one of which had an intensive and the other extensive herd management, were selected at random. In August 2001, a one-off collection of 93 rectal swabs of young animals was organised in those herds. Data on herd management and

health were recorded. The characteristics of the four herds investigated (A–D) are given in Table 1. In Herd A with commercial milk production, lambs were housed 25 per pen, and showed clinical symptoms of a diarrhoeic disease. Using sterile wads of cotton, rectal swabs of 32 lambs 1 to 3 weeks old were taken and placed into a transport medium (Cary-Blair medium, CM519, Oxoid). At the time of sampling, the lambs were receiving intensive chemotherapy with orally administered potentiated sulphonamides. In Herd B (commercial goat fattening farm), rectal swabs of 29 goats 2 to 6 months of age were taken; some goats showed symptoms of chronic diarrhoea. This herd was also being treated with sulphonamides. In the extensively managed Herd C, 14 lambs aged 1 to 3 months were selected for rectal swabs; some lambs occasionally suffered from diarrhoea. In extensively managed Herd D, rectal swabs were taken from 18 goats 2-months, with occasional diarrhoea. The rectal swabs taken were kept in a transport medium for 14 days at 6°C and shipped to the laboratory for processing in an isothermal box.

STEC isolation and identification. Rectal swabs samples were removed from the transport medium and enriched in EC broth with novobiocin (Okrend, 1990) overnight at 37°C without shaking. To demonstrate enterohemolysin production, they were subsequently directly cultivated on blood agar with washed ovine erythrocytes and CaCl₂ (Beutin et al., 1996b). At the same time, Dynabeads anti-*E. coli* O157 were used according to the manufacturer's instructions (Dynal A.S. Oslo, Norway) for immunomagnetic separation of 1 ml of the enriched sample. The immunobeads suspension was cultivated on Sorbitol MacConkey agar (CM813, Oxoid) with cefixime and potassium tellurite (CT-SMCA),

Table 1. Characteristics of farms and sheep and goat herds

Farm	Herd size	Breed	Management/efficiency	Type of housing	Feeding	Age group examined	Diarrhoea/antibiotics
A (sheep)	185	Awassi	intensive/milk production	pens, 25 animals	granulated mix, hay, alfalfa	lambs 1–3 weeks	Yes/Yes
B (goats)	100	local cross	intensive/fattening	free housing	granulated mix, occasional grazing	young goats 2–6 months	Yes/Yes
C (sheep)	100	local cross	extensive/dual purpose	free housing	year-round grazing	lambs 1–3 months	Yes/No
D (goats)	77	local cross	extensive/dual purpose	free housing	year-round grazing	young goats 2–8 weeks	Yes/No

and after a 24-hour incubation at 37°C, sorbitol-negative colonies were agglutinated with the *E. coli* O157 latex test kit (DR620, Oxoid). The serologically confirmed cultures of *E. coli* O157 were subcultivated on Nutrient Agar (CM3, Oxoid), confirmed by a technique described elsewhere (Cizek et al., 1999) and stored at –80°C in a cryoprotective medium (43.5 ml glycerol, 6.5 ml water, 50 ml 1.5% BactoPeptone) for further typing.

On blood agar with washed ovine erythrocytes and CaCl₂, suspect STEC non-O157 colonies were selected on the basis of the typical delayed haemolysis (Beutin et al., 1996b). Also selected were colonies where haemolysis occurred after 4 hours of incubation. The colonies were further subcultivated on Nutrient Agar (CM3, Oxoid), pre-confirmed by the beta-glucuronidase and tryptophanase activity testing (COLI test, Pliva – Lachema), and stored for a more detailed identification and typing at –80°C.

O and H antigen identification. Somatic O antigens were detected in *E. coli* cultures propagated on Nutrient broth No. 2 (Imuna, Slovak Republik) with 70 type antisera (Salajka et al., 1992). After an 18-hour cultivation at 37°C, the cultures were stained with triphenyltetrazolium chloride. Antigens were identified by agglutination on U-type microplates. Drops of antiserum diluted to the agglutination titre were pipetted to pits in the plate, and stained antigen boiled for 1 hour at 120°C was added. After shaking, platelets were kept at room temperature and results were read the following day. The serum agglutination titre was a reciprocal value of the dilution that led to complete agglutination. Tests for the presence of the H7 antigen were done with H7 specific antiserum (Denka Seiken, Japan) according to instructions of the manufacturer.

Virulence genes detection by PCR. The cultures were inoculated on Nutrient Agar (CM3, Oxoid), incubated at 37°C for 24 hours. A bacteriological loop was used to carry a part of the colony to 45 µl distilled water with 5 µl buffer for PCR, vortexed, boiled for 10 min, centrifuged for 2 min at 12 000 × g, and the supernatant was used for PCR. Multiplex PCR was used to test for the presence of *stx1*, *stx2*, *eaeA* and *ehlyA* genes (Paton and Paton 1998). The PCR method described elsewhere was used in the gene assay of thermolabile and thermostable enterotoxins (Alexa et al., 1997). PCR products were subject to electrophoresis in 2% agar gel at 120 V for 45 min, stained with ethidium bromide, visualized in UV light, and documented in a digital format.

Antibiotic susceptibility tests. Antibiotic susceptibility was tested by disk diffuse method on Mueller-Hinton agar (CM337, Oxoid) according to Bauer et al. (1966) and in accordance with National Committee for Clinical Laboratory Standards (NCCLS) Guidelines using 17 antibacterial substances: amoxicillin + clavulanic acid (AMC, 30 µg), ampicillin (AMP, 10 µg), streptomycin (S, 10 µg), apramycin (APR, 15 µg), neomycin (N, 30 µg), gentamicin (CN, 10 µg), amikacin (AK, 30 µg), colistin sulphate (CT, 10 µg), sulphonamides cp. (S3, 300 µg), trimethoprim-sulfamethoxazole (SXT, 25 µg), tetracycline (TE, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), nalidixic acid (NA, 30 µg), oxolinic acid (OA, 2 µg), cephalothin (KF, 30 µg), and ceftazidim (CAZ, 30 µg) (Oxoid, UK).

Multiresistant STEC O157:H7 isolate was tested by standard broth microdilution procedure and results were interpreted by using the criteria of the NCCLS. Broth microdilution panels were obtained from Trios, s.r.o., Czech Republic. The plates were read manually for growth to score the MIC determinations by using the NCCLS breakpoints.

For quality control purposes, the control strain *Escherichia coli* ATCC 25922 was run simultaneously with the test organisms.

STEC O157:H7 typing by RAPD. Primers of a known nucleotide sequence were used for isolate typing (Madico et al., 1995). After a primer confirmation, primers 1247 (AAGAGCCCGT) and 1254 (CCGCAGCCAA) were used for final typing. Boiling was used to isolate DNA, and the amount of DNA was measured by a spectrophotometer (SMART SPECTM 3000, Bio-Rad, USA). The PCR conditions were adjusted according to Galland et al. (2001). Amplified products were subjected to electrophoresis in 1% agarose at 100 V for 90 minutes. The resulting RAPD patterns were visualized in UV light, documented in a digital format and subsequently analyzed.

Data analysis. All data were entered into spreadsheets (Excel, Microsoft), which were used to calculate STEC and antimicrobial resistance prevalence.

RESULTS

A total 46 rectal swabs from lambs were tested. STEC carriers were identified in 25% and 57% of cases in the intensive and extensive management herds, respectively. Of the total of 32 lambs exam-

ined on Farm A with intensive milk production, STEC O157:H7 was demonstrated in 8 cases; on Farm C with extensive management only STEC of non-O157 serotypes were found (with the predominance of K85 and O128:K85 serotypes) in 8 of 14 lambs tested.

Of the total 47 young goats examined, STEC non-O157 were found in 28 cases. The STEC occurrence rates on the farms with extensive (D) and intensive (B) management were 50% and 65%, respectively. The isolates predominating among young goats were serogroups O128, O15 and O78. The occurrence of STEC O157 and non-O157, pathogenicity factors and resistance (R) types in STEC isolates on farms investigated are shown in Table 2. A PCR analysis of selected determinants representing virulence factors showed that all *E. coli* O157:H7 isolates featured genes coding Shiga toxin 2 (*stx2*), intimin (*eaeA*) and enterohemolysin (*ehlyA*). In all non-O157 STEC isolates, *stx1* and *ehlyA* genes were found. Alone *stx2* and *Stx* genes were

detected in two STEC unidentified serotypes from goat herd with extensive management.

Antibiotic susceptibility tests revealed that all eight lamb STEC O157:H7 isolates were resistant against one of the antibiotics tested with four different resistance profiles.

In one case, one isolate was multiresistant with resistance against ampicillin, ampicillin-sulbactam, cephalosporins (cefazolin, cefuroxime), monobactams (aztreonam), sulphonamides, co-trimoxazole, aminoglycosides, tetracycline and chloramphenicol. Furthermore the MICs of following antibacterial substances were obtained ($\mu\text{g/ml}$): ampicillin (> 32), ampicillin+sulbactam (32), cephalazolin (> 32), cefuroxime (64), cefoxitin (16), aztreonam (> 64), chloramphenicol (> 32), tetracycline (> 32), gentamicin (64), colistin (< 0.5), trimethoprim-sulfamethoxazole (> 128), ofloxacin (0.5). Compared with resistant STEC O157:H7 isolates, the multiresistant isolate had a different RAPD pattern (Figure 1).

Table 2. Prevalence and characteristics of STEC isolated

Farm	Animals positive/examined	Serogroups of STEC	PCR <i>stx/eaeA/hly</i>	R type ^c
A (sheep)	8/32 (25.0) ^a	O157:H7 (8) ^b	8 × <i>stx2/eaeA/hlyA</i>	1 × CSu
				5 × ACSSu
				1 × ACSuTp
				1 × ACCazAzGKfSSuTTP
B (goats)	19/29 (65.5)	O15 (2)	2 × <i>stx1/hlyA</i>	1 × SuTp
		O78 (3)	3 × <i>stx1/hlyA</i>	1 × ASSuTp
		O128 (2)	2 × <i>stx1/hlyA</i>	
		O128:K85 (2)	2 × <i>stx1/hlyA</i>	
		K85 (7)	7 × <i>stx1/hlyA</i>	
		OX (3)	3 × <i>stx1/hlyA</i>	
C (sheep)	8/14 (57.1)	O128:K85 (3)	3 × <i>stx1/hlyA</i>	
		K85 (5)	5 × <i>stx1/hlyA</i>	
D (goats)	9/18 (50.0)	O128:K85 (2)	2 × <i>stx1/hlyA</i>	
		K85 (5)	5 × <i>stx1/hlyA</i>	
		OX (2)	2 × <i>stx2/hlyA</i> ^d	

^apercentage of positive cases; ^bnumber of isolates; ^cR type, drugs to which isolates were resistant; ^dpositive assay of thermostable enterotoxin gene

A = ampicillin; C = chloramphenicol; Caz = ceftazidime; Az = aztreonam; G = gentamicin; Kf = cephalotin; S = streptomycin; Su = sulphonamide; T = tetracycline; Tp = trimethoprim-sulfamethoxazole (co-trimoxazole)

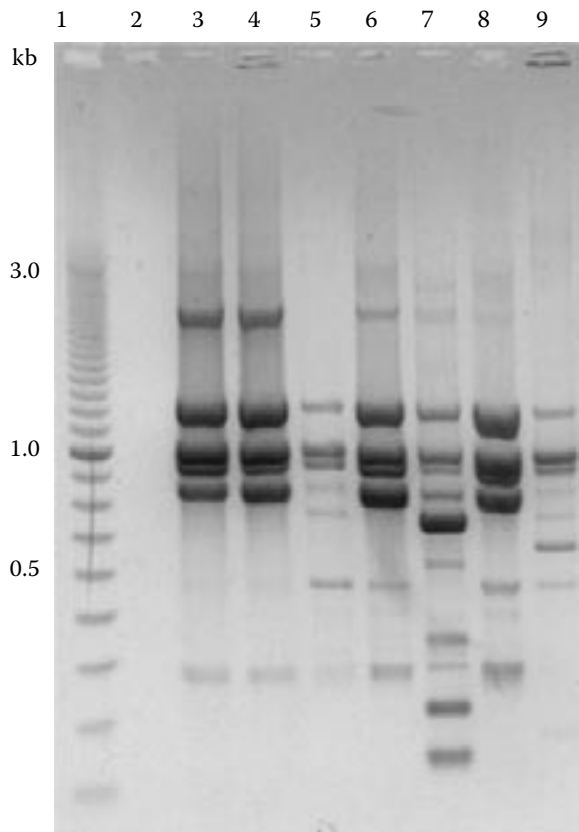


Figure 1. RAPD-PCR results for STEC O157:H7 isolates with different resistance profile

1-kb DNA ladder marker in lane 1. Negative control in lane 2. Lanes 3–6, four isolates with the same R type (ACSSu). Lane 7, multiresistant isolate (ACCazAzGKfSSuTTp). Lanes 8 and 9 resistant isolates with R type (ACSuTp) and (CSu), respectively. Less DNA of isolate in lane 5 could influence its evaluation

In the goat herd with intensive management, two resistant STEC O15 isolates were found. No resistant STEC strains in sheep and goat herds with extensive management were found.

DISCUSSION

There are many papers reporting the colonisation of the gastrointestinal tract of both large and small ruminants with STEC serotypes (Kudva et al., 1996; Blanco et al., 1997; Nielsen et al., 2002). The direct relationship between the STEC shed by small ruminants or the presence of the bacteria in their products on the one hand and human infections on the other has been, however, demonstrated only sporadically (Bielaszewska et al., 1997; Chapman et

al., 2000). A relatively frequent occurrence of the bacteria carriers among sheep (Kudva et al., 1996) and the STEC found in sheep and goat products (Rubini et al., 1999; Chapman et al., 2001; Chiueh et al., 2002) suggest that this may be a relatively important source of infection for people. This may be particularly true in regions with a high density of sheep and goat herds with many opportunities for contacts between people and small ruminants, where products of animal origin are processed by traditional methods and technologies and constitute a significant part of the local population diet per consumer basket. Recently published data on the incidence of both symptomatic and asymptomatic human infections caused by STEC O157 in northern Palestine (Adwan et al., 2002) underlined the importance of those infections in the Near East, where sources of those infections in people have been little investigated (Adwan and Adwan, 2004). Results of our pilot study are the first data on the prevalence of STEC carriers in sheep and goat herds with intensive and extensive herd management in Jordan. A particularly important finding was the demonstration of STEC O157:H7 in rectal swabs in lambs in a herd with commercial milk production. Such farms pose a real threat of milk contamination during milking, which may subsequently cause infections in people consuming raw milk or milk products. Neither should the possibility a contamination of water supply systems by faeces of carriers (Danon-Schaffer, 2001) be under-estimated.

Although the 3-week-old lambs examined suffered from diarrhoea, the results obtained in our study do not allow the conclusion that the diarrhoea was caused by STEC bacteria. Such conclusion would only be possible if other parasitic, viral or bacterial agents were excluded, and a histological examination of the mucous membrane of the lambs' colon made. The suggestion/suspicion that STEC O157:H7 might play a role in the onset of diarrhoea have been probably corroborated by results of experimental infections in neonatal calves reported by Dean-Nystrom et al. (1998). The cause of occasional diarrhoea in young goats in the extensively managed Herd D might be untypable STEC isolates that were, moreover, equipped with the gene for thermostable enterotoxin.

STEC O157 had previously been demonstrated in faeces of clinically healthy sheep (Kudva et al., 1996; Paiba et al., 2002). Experimental infections of young adult sheep have confirmed that STEC

O157 is better adapted for long-term persistence in the alimentary tract of sheep than other types of pathogenic *E. coli* (Cornick et al., 2000), which is due to presence of intimin (Cornick et al., 2002). In all STEC O157:H7 isolates in our study, the *eaeA* gene was demonstrated, and their long-term persistence in the sheep Herd A may therefore be assumed. The shedding of STEC in sheep is usually temporary and, as in cattle, subject to seasonal and dietary influences (Kudva et al., 1995, 1996, 1997). In this study, sampling took place in the hottest month of the year, when a high frequency of STEC shedding may also be expected.

The spectrum of STEC non-O157 serogroups isolated from goats was broader than from lambs, and the predominant serotype and serogroups were O128:K85, O15 and O78. These serogroups, or some of them, were previously demonstrated in sheep in, e.g. the USA (Kudva et al., 1997), Taiwan (Chiueh et al., 2002) Australia (Ramachadran et al., 2001) and Austria (Grif et al., 1999). Similar data on STEC serogroups in goats and on human infections caused by STEC non-O157 in the Middle East countries are however missing.

The presence of the *ehlyA* gene ascertained in all STEC non-O157 cultures isolated in this study was probably influenced by the approach to the selection of suspect STEC colonies that was governed by enterohemolysin production criteria. A similar approach to the STEC assay in cattle faeces has been used by Hornitzky *et al.* (2001). A higher incidence of the bacteria might have probably been found if another method of STEC screening had been used, which is clear from a comparison of our results and those reported by Fegan and Desmarchelier (1999).

It's not surprising that resistance to antimicrobials was found only in STEC isolated in intensively managed sheep and goat herds where the treatment was common. The finding of four different R types of STEC O157:H7 on the sheep Farm A is indicative of a selective pressure of several groups of antimicrobial substances on the farm related to treatment some time in the past. If sanitary regulations are not observed, the conditions on such farms are such that a transmission of resistant STEC strains to people, or at least of a transmission of multiresistance encoding determinants from coliform bacteria of animal origin to human strains, may take place (Oppegaard et al., 2001). The results obtained in the study again confirmed the fact that such risks are high in countries where an antibiotic control strat-

egy is missing and the use of antibiotics is not regulated. It has also been supported by recent findings in northern Palestine (Adwan et al., 2002; Adwan and Adwan, 2004) where 49% of human STEC isolates and 55% of STEC isolates from raw beef were found resistant to three or more antibiotics.

The use of antibiotics for the treatment of STEC infections may be contraindicated because certain antibiotics induce the release and dispersion of Shiga-toxin encoding bacteriophages (Zhang et al., 2000), or even complicate the development of clinical situation of patients (Wong et al., 2000).

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