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Dissemination of intestinal pathogens between lambs and puppies in sheep farms

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ABSTRACT

Objectives of the present work were (i) to confirm pathogens implicated in cases of diarrhoea in newborn and young lambs in sheep farms in Greece and (ii) to investigate a possible relation in dissemination of pathogens between lambs and dogs present in the farm. Work was carried out in 22 sheep farms, with (i) flock size over 150 animals, (ii) presence of clinical signs of diarrhoea in lambs in the flock and (iii) close and continuous contact and movement of shepherd dogs within the animal shed of each farm. Faecal sample collection from lambs was performed within 48 h of onset of clinical signs and prior to administration of any antimicrobial or antiparasitic medication to lambs. Faecal samples were also collected from puppies in the farm. In total, samples were collected from 126 lambs and 58 puppies. Samples were processed by using established techniques for isolation of bacteria, detection of viruses and observation of protozoan oocysts. *Escherichia coli* isolates obtained during the study, were tested for antimicrobial resistance against a variety of antimicrobial agents. In total, 236 bacterial isolates were recovered from faecal samples of lambs and 165 isolates from faecal samples of puppies. *E. coli* was the most frequently isolated microorganism: 104 isolates from lambs and 109 isolates from puppies were recovered. Other bacteria isolated were *Enterobacter* spp., *Proteus* spp., *Klebsiella* spp., (lambs and puppies), *Clostridium perfringens*, *Citrobacter freundi*, *Salmonella enterica* subsp. *diarizonae* (only lambs) and *Streptococcus* spp. (only puppies). Group A *Rotavirus* was detected in samples from lambs (2.5%) and *Parvovirus* in samples from puppies (5%). *Cryptosporidium* spp. oocysts were observed in samples from lambs and puppies. This is the first report of isolation of *S. enterica* subsp. *diarizonae* and of detection of *Rotavirus* from lambs in Greece. Rates of *E. coli* isolates from puppies resistant to antimicrobial agents were, in general, smaller than respective rates in isolates from lambs. Two pairs of isolates from the same farm (one from a lamb and one from a puppy) with identical patterns of resistance to antimicrobial agents were detected, which provides some evidence in support of a hypothesis that members of each pair might possibly have been spread from one animal species to the other.

1. Introduction

In newborn and young lambs, enteric infections are frequent problems, occurring in enzootic form or as acute outbreaks (Rook et al., 1990; Sargison, 2008). Enteric infections are caused by a variety of pathogens and are the predominant cause of neonatal mortality of lambs. They can cause clinical disease and/or may lead to suboptimal growth rate of affected animals (Sargison, 2008).

The role of bacteria and parasites in causation of the problem has been well documented. Among bacteria, *Escherichia coli*, *Salmonella* spp. and *Clostridium* spp. are the predominant pathogens causing clinical disease, although other organisms (e.g., *Campylobacter*)

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may be involved less frequently (Harp et al., 1981; Munoz et al., 1996; Scholes et al., 2007; Yang et al., 2014). Among parasites, *Cryptosporidium* spp. and *Giardia* spp. have now been recognised to be of significant involvement in the aetiology of the problem (Causapé et al., 2002; O'Handley and Olson, 2006; Geurden et al., 2008; Yang et al., 2009; Minetti et al., 2014). However, there is significantly less knowledge regarding a potential role of viruses, especially their implication as causative agents of the disease (Martella et al., 2015); rotaviruses, coronaviruses and adenoviruses could be of greater significance, although other viruses (e.g., picornaviruses, bunyaviruses) could also play a role in the problem (Lehmkuhl and Hobbs, 2008; Alkan et al., 2012; Chatzopoulos et al., 2013; Hubalek et al., 2014).

In establishing control measures for the syndrome, limiting the spread of potential causal agents is significant for effective management of disease. Transmission of intestinal microbial pathogens between sheep and dogs cannot be ruled out. There is extensive documentation regarding transmission of infective forms of parasites (helminthes or protozoa) between sheep and dogs (Vasileiou et al., 2015). However, there is no information regarding the potential role of dogs in the dissemination of enteric microbial pathogens in sheep flocks, although these animals are present in sheep farms around the world (bar in intensively managed flocks). Dogs also are in close proximity with humans, hence pose an increased risk to transmit zoonotic pathogens.

Objectives of the present work were (i) to identify and confirm pathogens implicated in cases of diarrhoea in newborn and young lambs in sheep farms in Greece and (ii) to investigate a possible relation in dissemination of intestinal microorganisms between lambs and dogs present in the farm.

2. Materials and methods

2.1. Study design and sampling strategy

Work was carried out from late autumn 2014 to early spring 2015, during the lambing and lactation period in 22 sheep farms in central Greece. A variety of indigenous (e.g., Karagouniko, Chios) or imported (Lacaune, Assaf) breed animals were present in the flocks. As per principal production system in Greece, lambs sucked their dams until 45- to 55-day-old, at which age they would go for slaughter. Sheep management in these farms was of the semi-intensive or semi-extensive type.

Inclusion criteria for flocks were (i) flock size over 150 animals, (ii) presence of clinical signs of diarrhoea in lambs in the flock and (iii) close and continuous contact and movement of shepherd dogs within the animal shed of each farm.

Faecal sample collection from lambs was performed within 48 h of onset of clinical signs and prior to administration of any antimicrobial or antiparasitic medication to lambs. Then, faecal samples were collected from puppies younger than 45 days present in the farm, which had not been vaccinated and had not received any anthelmintic treatment.

In total, faecal samples were collected from 126 newborn lambs younger than two weeks. Within each flock, samples were collected from three to six lambs. Additionally, samples were collected from 58 puppies living in the same farms, two to four puppies in each farm. Initially, a swab was inserted (1.0–1.5 cm) into the rectum of each animal, swirled and then removed. Further, 20 g of faeces were collected into the gloved hand of the investigator. Swabs were placed into transport medium and faeces were maintained in cold storage until transport to the laboratory, which took place within 4 h maximum.

2.2. Laboratory examinations

In all cases, rectal swabs and faecal samples were processed within 24 h of collection. Samples were processed for presence of microbial or parasitic agents by using the techniques described below.

2.2.1. Bacteriological tests

At first, rectal swabs were cultured onto 5% sheep blood agar and McConkey agar and incubated at 37 °C for up to 48 h. Morphology of colonies on each plate was examined; all colonies on the same plate found to be morphologically different between them, were cultured on tryptic soya agar to recover pure colonies. Conventional microbiological techniques, the API rapid identification system (Biomerieux, Marcy-l'-Etoile, France) and the automated identification Vitek 2 system with card GN (Biomerieux) were used for identification of organisms. Swabs were also cultured on CDC Anaerobe 5% Blood Sheep agar (Becton-Dickinson, Franklin Lakes, NJ, USA) for anaerobic incubation up to 72 h for possible isolation of *Clostridium perfringens*. For isolation of *Salmonella* spp., the procedure described in ISO: 6579:2002 protocol was followed, starting with a 20 g of faeces and using Buffered Peptone Water as recovery medium, a Modified Semisolid Rappaport-Vassilliadis Medium and a Xylose-Lysine Desoxycholate agar agar.

For identification of *E. coli*, the following characteristics were primarily taken into account: growth onto McConkey agar (pink coloured colonies with bright pink halo), results of Gram stain (Gram negative rod-shaped), evaluation of bacterial motility (motile organisms), results of lactose fermentation (lactose fermenting), of D-glucose fermentation (glucose fermenting with gas production), of D-mannitol fermentation (mannitol fermenting) and of sorbitol fermentation (sorbitol fermenting) and result of indole production test (indole producing), of Voges/Proskauer test (negative result), of urea hydrolysis test (non-hydrolysing organism), of H_2S production test (non-H2S producing organism), of aesculin hydrolysis (non-hydrolysing organism), of lysine decarboxylase test (positive test), of citrate ulitisation test (negative result) and of ONPG test (positive result) (Barrow and Feltham, 1993; Edens et al., 1997).

2.2.2. Virological tests

For detection of Group A *Rotaviruses*, *Adenovirus*, *Coronavirus* and *Parvovirus* in faecal samples, commercially available rapid test kits (VIKIA Rota-Adeno; Biomerieux—Rota-Corona-Parvo Quicking; Quicking, Shanghai, China), which detected viral antigens, were used, as per manufacturers' specifications. When *Rotavirus* was detected in a sample, the presence was confirmed by using a RT-PCR protocol as previously described (World Health Organization, 2009); faecal homogenates were prepared in phosphate buffer saline and RNA extraction was performed using a commercially available RNA kit (Ambion RNA kit; Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturers' protocol. The extracted double stranded RNA was denaturated at 97 °C for 5 min and placed immediately on ice; a two-step multiplex RT-PCR was performed to characterise G and P types of the *Rotavirus* strains, based on previously published terms and conditions (World Health Organization, 2009); amplicons corresponding to each G and P type were visualised under ultra-violet light on 2% agarose gel.

2.2.3. Detection of Cryptosporidium spp.

For detection of *Cryptosporidium* spp. in faecal samples, a smear from each sample was stained by means of the modified Ziehl-Neelsen technique; additionally, the flotation method using ZnSO4 33.2% solution was performed to detect any *Giardia* spp. oocysts (Ministry of Agriculture, Fisheries and Food, 1986).

Table 1
Frequency of isolation of bacteria from faecal samples of lambs with diarrhoea, in sheep farms in Greece.

	Isolates		Samples		Farms	
	n	%	n	%	n	%
Escherichia coli	104	44%	80	63.5%	22	100%
Enterobacter aerogenes	39	16.5%	39	31%	8	36.5%
Proteus vulgaris	23	9.5%	23	18.5%	9	41%
Klebsiella pneumoniae	20	8.5%	20	16%	8	36.5%
Proteus mirabilis	16	7%	16	12.5%	7	32%
Clostridium perfringens	14	6%	14	11%	3	13.5%
Citrobacter freundii	9	4%	9	7%	3	13.5%
Klebsiella oxytoca	4	1.5%	4	3%	1	4.5%
Salmonella enterica subsp. *diarizonae*	4	1.5%	4	3%	1	4.5%
Enterobacter cloacae	3	1.5%	3	2.5%	1	4.5%
Total	236	100%	126	100%	22	100%

2.2.4. *Antimicrobial susceptibility of* Escherichia coli *isolates*

All *E. coli* isolates obtained from lambs or puppies were tested for antimicrobial susceptibility against the following: amikacin, amoxicillin/clavulanic acid, ampicillin, aztreonam, cefepime, cefotaxime, cefoxitin, ceftazidime, ciprofloxacin, imipenem, meropenem, piperacillin, piperacillin/tazobactam, ticarcillin, ticarcillin/clavulanic acid and trimethoprim-sulfamethoxazole. The work was performed in the automated system Vitek-2 with cards xn01 and n233 (Biomerieux).

2.3. Data management and analysis

E. coli isolates were classified as susceptible, of intermediate susceptibility or resistant based on cut-off values adopted by the European Committee on Antimicrobial Susceptibility Testing and breakpoints according to VET01-A4 standard (Clinical and Laboratory Standard Institute, 2013).

Rates of resistance to antimicrobial agents in isolates from lambs or puppies were compared between them by calculating the z-ratio for the significance of the difference between two independent proportions (Lowry, 2012) in an electronic data management tool (Lowry, 2015).

3. Results

3.1. Clinical findings

Clinical severity of diarrhoea in lambs was of mild intensity to profuse, intense disease with dehydration of the animal. Median morbidity rate in flocks into the study was 15% (range: 10%–60%); median fatality rate was 12% (range: 0%–40%). In puppies, occasionally, soft-type faeces were recorded, but in no case diarrhoea was evident in any of the sampled animals.

3.2. Pathogen detection in samples from lambs

In total, 236 bacterial isolates were recovered from faecal samples of lambs.

E. coli, in pure or mixed culture, was the most frequently isolated microorganism: 104 isolates were recovered from 70 lambs (one isolate from 42 lambs, two isolates from 22 lambs and three isolates from 6 lambs), i.e. mean number of *E. coli* isolates per lamb was 0.83 ± 0.08. Other bacteria isolated were *Enterobacter* spp., *Proteus* spp., *Klebsiella* spp., *Cl. perfringens*, *Citrobacter freundii* and *S. enterica* subsp. *diarizonae* (Table 1).

Group A *Rotavirus* was detected in samples from three lambs (2.5%) in two different flocks (9%); in both flocks, reported morbidity rate was increased (45% and 60%, respectively, in each of the two flocks) and fatality rate small (<5% in both flocks); the G10P[8] combination was determined in one sample, i.e. regarding one *Rotavirus* strain, while the other two *Rotavirus* strains (both from the same flock) remained untyped. *Coronavirus*, *Adenovirus* or *Parvovirus* were not detected in any sample from any flock. Finally, *Cryptosporidium* spp. oocysts were observed in smears of faecal samples from 13 lambs (10.5%), collected from 11 flocks (50%), whilst no *Giardia* oocysts have been detected.

Table 2
Frequency of isolation of bacteria from faecal samples of puppies, in sheep farms in Greece, where lambs with diarrhoea were present.

	Isolates		Samples		Farms	
	n	%	n	%	n	%
Escherichia coli	109	66%	57	98.5%	22	100%
Enterobacter aerogenes	18	11%	18	31%	12	54.5%
Klebsiella pneumoniae	15	9%	26%	26%	7	32%
Proteus vulgaris	14	8.5%	14	24%	9	41%
Streptococcus canis	6	3.5%	10.5%	10.5%	2	9%
Streptococcus dysgalactiae	3	2%	5%	5%	2	9%
Total	165	100%	58	100%	22	100%

Table 3
Frequency of concurrent isolation of organisms from faecal samples from lambs with diarrhoea and from puppies in the same sheep farm (all farms in Greece).

	Farms	
	n	%
Escherichia coli	22	100%
Enterobacter aerogenes	8	36.5%
Proteus vulgaris	6	27.5%
Klebsiella pneumoniae	3	13.5%
Cryptosporidium sp.	1	4.5%
Total	22	100%

3.3. Pathogen detection in samples from puppies in sheep farms

In total, 165 bacterial isolates were recovered from faecal samples of puppies in the sheep farms.

E. coli, in pure or mixed culture, was the most frequently isolated microorganism: 109 isolates were recovered from 57 puppies (one isolate from 18 puppies, two isolates from 27 puppies and three isolates from 11 puppies, four isolates from 1 puppy), i.e. mean number of *E. coli* isolates per puppy was 1.88 ± 0.10 ($P < 0.001$ when comparing mean number of isolates per lamb and per puppy). Other bacteria isolated were *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp. and *Streptococcus* spp. (Table 2).

Parvovirus was detected in faecal samples from 3 puppies in one farm. No other virus was detected in any sample from puppies. Finally, *Cryptosporidium* spp. oocysts were observed in smears of faecal samples from 3 puppies (5%), collected from 3 farms (1.5%), whilst also no *Giardia* oocysts have been detected.

3.4. Concurrent pathogen detection in samples from lambs or puppies in the same farm

E. coli, *E. aerogenes*, *P. vulgaris*, *K. pneumoniae* and *Cryptosporidium* sp. were recovered concurrently from samples from lambs or puppies in the same farm (Table 3).

3.5. *Antimicrobial susceptibility of* Escherichia coli *isolates*

Antibiotic susceptibility tests revealed 14 and 9 different patterns of antibiotic resistance in the isolates recovered from lambs or puppies, respectively.

Of isolates from lambs, 63 (60.5%) were found resistant to at least one antimicrobial agent and 35 (33.5%) to at least three agents classified into three different groups. Resistance to a β-lactam

Table 4
Presentation of two patterns of resistance to antimicrobial agents, which were identical in *E. coli* isolates recovered from a lamb with diarrhoea and a puppy in the same farm.

	Farm ID 3		Farm ID 11	
	Lamb	Puppy	Lamb	Puppy
Amikacin	S	S	S	S
Amoxycillin/Clavulanic acid	R	R	S	S
Ampicillin	R	R	R	R
Aztreonam	S	S	S	S
Cefepime	S	S	S	S
Cefotaxime	S	S	S	S
Cefoxitin	S	S	S	S
Ceftazidime	S	S	S	S
Ciprofloxacin	S	S	S	S
Imipenem	S	S	S	S
Meropenem	S	S	S	S
Piperacillin	R	R	I	I
Piperacillin/Tazobactam	S	S	S	S
Ticarcillin	R	R	R	R
Ticarcillin/Clavulanic acid	R	R	R	R
Trimethoprim/Sulfamethoxazole	R	R	R	R

R: resistant, S: sensitive.

antibiotic (ampicillin, piperacillin or ticarcillin) was detected in 45 (43.5%) isolates; finally, resistance to fluoroquinolones was detected in 7 (6.5%) isolates recovered from samples collected in two different flocks.

Of isolates from puppies, 56 (51.5%) were found resistant to at least one antimicrobial agent and 26 (24%) to at least three agents classified into three different groups ($P = 0.081$ and $P = 0.057$, when compared to respective proportions in isolates recovered from lambs). Resistance to a β-lactam antibiotic was detected in 30 (27.5%) isolates; finally, resistance to fluoroquinolones was detected in 2 (2%) isolates recovered from samples collected in the same farm ($P = 0.008$ and $P = 0.038$, when compared to respective proportions in isolates recovered from lambs).

Two pairs of isolates (one isolate from a lamb and one isolate from a puppy), each from the same farm, with identical patterns of resistance to antimicrobial agents were detected (Table 4).

4. Discussion

A variety of microorganisms have been isolated from faecal samples from lambs with diarrhoea, which implicates these organisms in aetiology of the syndrome, especially when the respective organisms have been isolated in pure culture. These results are in accord with previous findings in the literature (Manser and Dalziel, 1985; Adesiyun et al., 2001; Alvseike and Skjerve, 2002; Greco et al., 2005; Edrington et al., 2009; Osman et al., 2013; Ozmen et al., 2006; Wani et al., 2013; Gencay, 2014; Paz e Silva et al., 2014). Isolation in mixed culture from many samples may further indicate a potential synergistic role of these organisms. In fact, it has been previously documented that some of these microorganisms constituted the main part of intestinal flora of healthy animals and might, under various circumstances, become pathogenic for the hosts (Canny and McCormick, 2008).

In contrast, isolation of *S. enterica* subsp. *diarizonae* from lambs of one flock has indicated an outbreak of disease caused by that organism, which is a pathogen of wild boar and reptiles (Chiari et al., 2013; Marin et al., 2013; Wikström et al., 2014; Touloudi et al., 2015). Environmental infection into the farm barns might have led to subsequent infection of the newborns. In general, this organism is responsible for a considerable proportion of all *Salmonella* infections in lambs and can lead to significant losses (Davies et al., 2001; Alvseike and Skjerve, 2002). The organism has also been implicated as a cause of reproductive losses in ewes (Linklater, 2000). This is the first report of *S. enterica* subsp. *diarizonae* isolation from sheep in Greece, which indicates this pathogen as an emerging animal health problem in the country, with potential zoonotic implications (Sörén et al., 2015).

The present results confirm a remarkable role of Group A *Rotavirus* in lambs. Several studies worldwide have reported isolation of Group A and Group B *Rotavirus* in lambs, with a prevalence ranging from 1% to 40% (Theil et al., 1995; Wani et al., 2004; Khafagi et al., 2010; Gazal et al., 2011), whilst in clinically healthy lambs prevalence of antibodies against *Rotavirus* in blood serum samples may reach up to 40% (Bridger, 2008). In other animal species, pathogenicity of rotaviruses varies, depending mainly on the viral strain and the immunological status of the host, with outbreaks caused by the virus being a frequent feature of the disease (Galindo-Cardiel et al., 2011; Alkan et al., 2012). To our knowledge, this is the first report of a case of diarrhoea in lambs in Greece associated with *Rotavirus*. The G10 genotype is one of the most common determined VP7 types in ruminants, while P[8] is less frequently detected (Martella et al., 2010; Papp et al., 2014).

Pathogenicity of *Rotavirus* varies, depending mainly on the viral strain and the immunological status of the host, with outbreaks caused by the virus being a frequent feature of the disease (Galindo-Cardiel et al., 2011; Alkan et al., 2012). Rotavirus-caused diarrhoea in lambs, as well as in all susceptible hosts, is the outcome of several combined factors and events, which include changes in small intestinal homeostasis, alterations in epithelial surface area and finally increased integrity, necrosis or apoptosis of intestinal epithelial cells (Lundgren and Svensson, 2001). Death may occur in long-term cases of irrepressible diarrhea due to extended losses of proteins and electrolytes inbalance. Possibly, *Rotavirus* infection might have been the cause for the increased morbidity and reduced fatality observed in the two respective flocks, in samples from which the virus was detected.

Results of antibiotic susceptibility testing of *E. coli* isolates have indicated increased resistance and wide distribution of resistance patterns in isolates from both lambs and puppies (McEwen and Fedorka-Cray, 2002; Vantarakis et al., 2006; Mazurek et al., 2013). However, prevalence of resistance was smaller among isolates from puppies than among those from lambs; there was also evidence that rate of resistance specifically to β-lactams and fluoroquinolones was significantly smaller in isolates from puppies (Carattoli, 2008; Umber and Bender, 2009; Leonard et al., 2012; Wu et al., 2013). Obviously, frequent administration of antibiotics to newborn lambs as a means to control outbreaks of diarrhoea, is the primary contributing factor to this increased prevalence. In contrast, administration of antibiotics to puppies in farms is performed infrequently if at all. Thus, one might have assumed that antibiotic resistance in isolates from puppies would have been limited.

The detection of two pairs of *E. coli* isolates with identical patterns of antibiotic resistance/susceptibility is supportive of a hypothesis that members of each pair might possibly have been spread from one animal species to the other (Kaesbohrer et al., 2012). In association with this finding, the unexpectedly increased rate of antibiotic resistance in strains from dogs indicates that dissemination of strains would have occurred more likely from sheep to dogs. The results could point out that dissemination of intestinal microbes might possibly take place between sheep and dogs.

Sheep and dogs share the same environment in farms and roam across the same territories. Hence, it is possible that bacteria may spread from one species to the other. Dissemination of infective forms of parasites between dogs and sheep in a farm environment has been well documented (Vasileiou et al., 2015), whilst dissemination of bacterial pathogens between sheep and wildlife also occurs often (Billinis, 2013). In the study, puppies were chosen for sampling for two reasons: (i) their immune system would not be fully functional, hence they might have become infected and would

be excreting intestinal bacteria and (ii) their habit to roam inside sheep sheds and to enter lambing pens and/or growing lamb pens.

Other bacteria, *E. aerogenes*, *K. pneumoniae* and *P. vulgaris*, have also been isolated in samples from dogs and sheep in the same farms. In contrast, no evidence of dissemination between sheep and dogs has been found for other pathogens, e.g., *Salmonella* or *Rotavirus*. As discussed above, *Salmonella* spreads from reptiles and wild boar, which lead to dissemination of the organism in the environment and then to infection of grazing sheep. Rotaviruses are ubiquitous in livestock, remaining a constant health threat. Additionally, several *Rotavirus* strains have been shown to have the capacity to cross the species-barrier, thus infecting other animal species or humans. Unlike bovine *Rotavirus* strains, which have been repeatedly detected in samples from various animal species and humans, sofar there have been no similar reports regarding ovine isolates. However, features and epidemiology of ovine *Rotavirus* remain inadequately studied and poorly understood.

5. Concluding remarks

A variety of microbial pathogens was detected in samples from lambs with diarrhoea. *E. coli* was the predominant microbial pathogen, although other organisms were detected, among them, for the first time in sheep farms in Greece, *S. enterica* subsp. *diarizonae* and Group A *Rotavirus*. On two occasions, identical patterns of resistance to antimicrobial agents between isolates of the organism from lambs or puppies living in the same farms (i.e., two pairs of organisms) were found; this finding provides evidence for possible dissemination of enteric pathogens between sheep and dogs within the same farm. It is also noteworthy that, as dogs are in closer contact with humans than sheep are, this may lead to the indirect spread of ovine strains of pathogens to humans (e.g., antibiotic resistant strains).

Conflict of interest

The authors declare that there are no conflicts of interest.

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