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Research Article

DNA Hybridization of *Escherichia coli* Strains Isolated from Uteri and Fecal Samples of Bitches with Pyometra

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Abstract: *Escherichia coli* is the most common bacterium that has been isolated from the bacterial culture of uterine and fecal samples of dogs with pyometra. The aim of the present study was to determine whether this organism could be relevant to the pathogenesis of pyometra in dogs.

Fecal and uterine samples were collected from 17 bitches with pyometra. *E. coli* strains were isolated in all samples. Representative colonies of *E. coli* from each sample were analyzed for pathogenicity determinants by hybridization with HRP-conjugated DNA probes for enteropathogenic (*eae*A), verotoxigenic (VT1, VT2), enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteroaggregative (EAggEC) and enteroinvasive (EIEC) *E. coli*. A verocell assay for cytotoxic necrotizing factor (CNF) was performed. Fecal analyses showed that the dogs were excreting pathogenic *E. coli* that hybridized with probes for *eae*A (47%), VT1 (29%) and EIEC (17%) compared with the uterine samples in which both *eae*A and VT1 (41%), and EIEC (5%) positive *E. coli* were found. All samples were negative for VT2, EHEC, ETEC and EAggEC probes. A verocell assay confirmed the presence of CNF in the positive samples from feces (29%) and uterine samples (17%). CNF-positive samples from dogs with pyometra were also positive for CNF in fecal samples.

These findings suggest that pathogenic *E. coli* may play an important role in pyometra and the strains isolated from the uteri may be similar to isolates from feces. Further studies are needed to examine their potential role in the pathogenesis of pyometra in dogs.

Key Words: *Escherichia coli*, dog, pyometra, DNA hybridization

Dişi Köpeklerin Dışkı ve Pyometra Örneklerinden İzole Edilen *Escherichia coli*'lerin DNA Hibridizasyonu İle İncelenmesi

Özet: Köpeklerin dışkı ve pyometra örneklerinden en sık izole edilen bakteri *Escherichia coli*'dir. Çalışmanın amacı bu mikroorganizmanın köpeklerin pyometrasının patogeneziyle bir ilişkisinin olup olmadığını belirlemektir.

Dışkı ve pyometra örnekleri 17 dişi köpekden toplandı. *E. coli* bütün dışkı ve pyometra örneklerinden izole edildi. Her örnekten alınan *E. coli*'lerin patojenik özelliklerini belirlemek için HRP ile bağlanmış enteropatojenik (*eae*A), verotoksijenik (VT1, VT2), enterohemorajik (EHEC), enterotoksijenik (ETEC), enteroaggregativ (EAggEC) ve enteroinvasiv (EIEC) DNA probları ile hibridizasyonları yapıldı. Vero hücreleriyle de "Cytotoxic necrotizing factor" (CNF) özelligini belirlemek için bir test uygulandı.

Dışkı örneklerinin hibridizasyonu sonucu patojenik *E. coli* genleri; *eae*A (%47), VT1 (%29) ve EIEC (%17) bulunurken pyometra örneklerinin hibridizasyonu sonucu ise EIEC (%5), *eae*A ve VT1 (%41) genleri tespit edildi. Bütün dışkı ve pyometra örnekleri VT2, EHEC, ETEC veya EAggEC problarıyla negatif sonuç verdi. Pyometra örneklerinden izole edilen ve hibridizasyon testi ile pozitif sonuç veren *E. coli* suşları aynı zamanda dışkıdan da izole edildi. Vero hücreleriyle yapılan testte dışkı (%29) ve pyometra örneklerinde (%17) *E. coli* suşu CNF pozitif olarak belirlendi. Pyometra örneklerinden izole edilen CNF pozitif *E. coli* suşları aynı pyometralı köpeğin dışkısından da izole edildi.

Bulgular patojenik *E. coli*'nin köpeklerde pyometra oluşumunda önemli bir rol oynayabileceğini ve köpeğin kendi dışkısından da köken alabileceğini göstermektedir. Pyometranın patogenezini belirlemek için daha ileri çalışmalar gerekmektedir.

Anahtar Sözcükler: *Escherichia coli*, köpek, pyometra, DNA hibridizasyonu

Introduction

Escherichia coli is a major component of the intestinal flora in human beings and other warm-blooded animals (1,2). It has long been recognized that different strains of *E. coli* may cause enteric or non-enteric disease in dogs and many other species. The importance of *E. coli* has become more apparent as our understanding of the pathogenesis has increased, which is aided by recent advances in molecular biology (2-4). On the basis of virulence markers, enteric *E. coli* strains that cause disease in human beings and most warm-blooded animals are now classified into 5 major categories (5,6): Enterotoxigenic *E. coli* (ETEC), verotoxigenic or enterohemorrhagic *E. coli* (VTEC or EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAggEC). However, differences occur in categorization because there is considerable overlap in these definitions with the virulence mechanisms of the distinct pathogenic *E. coli* strains (for example, between VTEC and EHEC). In addition, it has recently been shown that some *E. coli* strains are able to elaborate 2 types of cytotoxic necrotizing factors (CNFI and CNFII), also named necrotoxigenic *E. coli* (NTEC) (7). These 2 toxins are dermonecrotic protein toxins produced by human and animal clinical isolates of *E. coli*; however, their role is still unclear (7-9). *E. coli* was the most frequently isolated bacterium from uterine samples (10-14). Some earlier studies pointed to the significance of *E. coli* in association with endometritis and pyometra in the bitch (15,16). Other early reports suggested that pyometra cases in dogs were associated with only a few strains belonging to classical human *E. coli* serotypes (17-20). Especially in bitches with urinary tract infection (UTI), *E. coli* were frequently isolated from infections of the uterus (21). It was suggested that subclinical UTI is associated with pyometra and that receptors for *E. coli* are developed in the endometrium and myometrium, thus enhancing the colonization of bacteria in the uterus (22). However, neither the strains nor the associated clinical pathological lesions from fecal and pyometra samples from the same bitch were extensively characterized. Therefore, the prevalence and relevance of *E. coli* causing pyometra in dogs is not well understood.

The aim of this study was to determine whether the strains of *E. coli* isolated from uterine and fecal samples of bitches with pyometra are identical by using DNA hybridization.

Materials and Methods

In the present study, fecal and uterine samples were collected from 17 bitches with pyometra that had previously been studied by Dhaliwal et al. (12). Samples were plated on MacConkey agar (Oxoid Ltd, Basingstok, UK). *E. coli* strains were isolated from all the samples. From each dog, 5 colonies with the typical appearance or with different morphologies of *E. coli* were chosen. An API test (API 20E System, BioMérieux SA, Mercy-l'Etoile, France) was carried out for identification of *E. coli*. Strains were stored in cryo vials (Protect, lab M) at -80 °C for hybridization assay.

Plasmids and control strains

Isolated representative colonies of *E. coli* from each sample were analyzed for pathogenicity determinants by hybridization with HRP-conjugated DNA probes for enteropathogenic (*eae*A), verotoxigenic (VT1, VT2), enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteroaggregative (EAggEC) and enteroinvasive (EIEC) *E. coli*. In addition, CNF positive strains of *E. coli* were tested for possible cross-reactions with any probe. The derivation of gene probes is shown in Table 1 and control strains used for the hybridization studies are shown in Table 2.

DNA hybridization assay

Probes were derived from the recombinant plasmids after purification by ultracentrifugation in cesium chloride-ethidium bromide density gradient (36). Appropriate restriction endonucleases were used to derive the gene probe fragment from the recombinant plasmid (Table 1). Fragments were separated by use of electrophoresis in an agarose gel and the DNA fragments were purified from gel.

For colony blots, 5 isolated *E. coli* strains from each sample were inoculated onto MacConkey agar (Oxoid) plates and incubated at 37 °C overnight. The wild strains of *E. coli* and control strains of DNA probes were transferred to 96-well plates (Falcon) containing 100 µl of sterile Luria broth base (LUB) (Gibco) per well. Each plate was replicated onto nylon filters (7 by 11 cm, Hybond-N+, Amersham, RPN 203B). The immobilization of *E. coli* colonies onto filters was performed as described previously (36). Following this, the filters were immersed in proteinase K solution (23). Colony hybridizations were performed in a hybridization oven (Hybridiser FHB-1DE, Techne Ltd, UK) with the enhanced chemiluminescence gene detection system (ECL; Amersham International plc,

Table 1. Derivation of the 7 gene probes.

Probe name	Plasmid	Endonuclease	Fragment (bp)	Reference
ETEC (LTh, ST1a, ST1b)	pKAD008	*Xba* I	1268	23
EaeA	pCVD434	*Sal* I + *Kpn* I	1000	24
EIEC	H1(60R706)	*Eco*R I + *Sal* I	1000	25
EHEC	pCVD419	*Hind* III	3400	26
VT2	pACYC184	*Sma* I + *Pst*I	850	27
EAggEC	pCVD432	*Eco*R I + *Pst* I	700	28
VT1	pACYC177	*Hinc* II	750	29

Table 2. Control strains of *E. coli* used in this study.

Class	Strain	Description	Reference or source
EAggEC	Wilmott	human EAggEC strain	J. W. S*
	Lewis	human EAggEC strain	J. W. S*
	Hoque	human EAggEC strain	J. W. S*
	17-Şub	from a child with diarrhea in Santiago, Chile	28
VTEC	E30480	serotype O157:H7, contains $VT1^+$, $VT2^+$, *eae*A	30
	E32511	serotype O157:H- contains $VT2^+$ and *eae*A	30
ETEC	E5798	serotype O7:H18, contains LT^+	31
	E2985	serotype O159:H34, contains ST^+, LT^+	31
	E7476	O166:H27, contains ST^+	31
	B44	serotype O9:K30:K99, contains ST^+	32
EPEC	E2348/69	serotype O127:H6 implicated in an outbreak of infant diarrhea in England	33
	RDEC-1	serotype O15:NM, rabbit strain	34
EIEC	EI314	serogroup O124	35

*kindly supplied by Prof. John Walker-Smith, Paediatrics and Gastroenterology, Medical College of St. Bartholomew's Hospital.

Amersham, RPN 3001) according to the manufacturer's instructions.

Cytotoxicty test

Verocells were used for the modified cytotoxicity assay as previously described (37). For CNF, changes occurred at 24-48 h when the affected cells appeared enlarged and multinucleated, and the cell sheet resembled a mosaic after staining with Giemsa.

Pathogenic *E. coli* strains isolated from dogs were compared by Fisher's exact test. Significance was accepted at $P \leq 0.05$.

Results

Fecal analyses showed that the dogs were excreting pathogenic *E. coli* strains that hybridized with probes for *eae*A (n = 8, 47%), VT1 (n = 5, 29%) and EIEC (n = 3, 17%) compared with the uterine samples in which both *eae*A and VT1 (n = 7, 41%), and EIEC (n = 1, 5%) positive *E. coli* were found. The X-ray film of the hybridization filters is shown in the Figure. All samples were negative for VT2, EHEC, ETEC and EAggEC probes.

The percentages of positive *E. coli* isolates detected with the gene probes for specific pathogenicity determinants are shown in Table 3. There were no significant differences between the gene probes for the specific pathogenicity determinants ($P > 0.05$). Hybridization assays determined that the 5 VT1 positive uterine samples and 4 VT1 positive fecal samples were also carrying the *eae*A gene.

In addition, a verocell assay confirmed the presence of CNF-positive *E. coli* strains. CNF-positive strains were

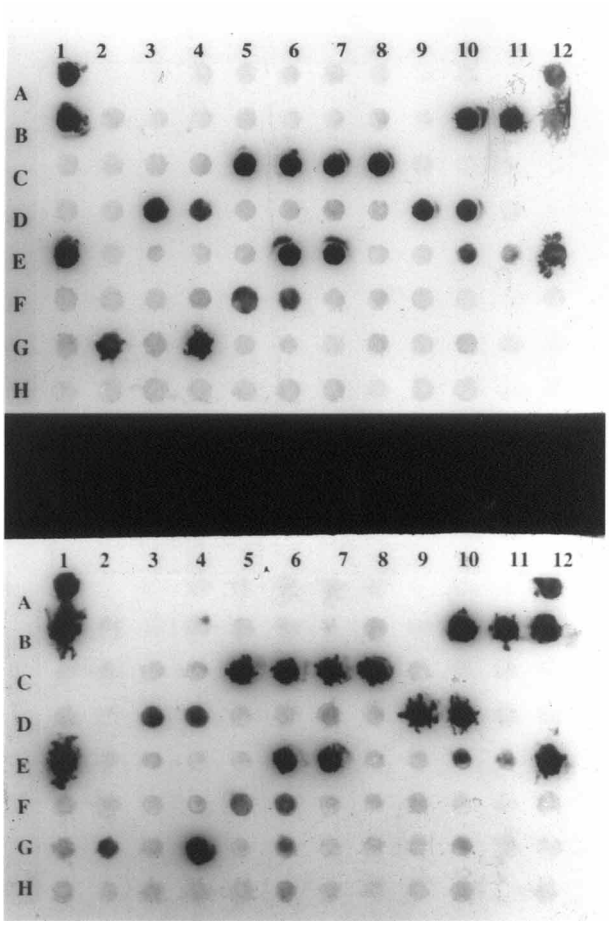

Figure. Colony hybridization with HRP-conjugated *eae*A probe of *E. coli* strains isolated from fecal and uterine samples. Positive control strains are located in duplicate: A1, A12 (E2348/69); B1, B12 (E30480) and E1, E12 (E32511). Others are the positive *E. coli* strains.

Table 3. Number of positive *E. coli* isolates detected with the gene probes and verocell assay for specific pathogenicity determinants.

Samples	VT1	EIEC	*eae*A	CNF
Fecal	5	3	8	4
(n = 17)	-%29	-%17	-%47	-%29
Pyometra	7	1	7	3
(n = 17)	-%41	-%5	-%41	-%17

isolated from 3 (17%) dogs with pyometra and from the fecal samples of 4r (29%) dogs. All 3 dogs with CNF-positive uterine samples also produced CNF-positive fecal samples. Giemsa staining of the plates determined these CNF-positive strains, which demonstrated characteristic changes for CNF during the verocell assay.

Discussion

E. coli is isolated most frequently from fecal samples from dogs and from infected uteri of bitches with pyometra. In a number of studies *E. coli* was isolated from 43 (90%) of the 48 uteri with pyometra (11) and again from bitches with pyometra *E. coli* in pure culture was the most frequent isolate (10,13).

In the present study, the most frequently identified *E. coli* strains in fecal and pyometra samples of dogs were EPEC and VTEC. It is evident from the results of the hybridization assays that there are genetic similarities between these isolates. In this study, the hybridization assay for *eae*A and VT1 was positive in both the uterine and fecal samples of dogs. In another study, *E. coli* strains from the uteri of bitches suffering from pyometra were examined and their DNA profiles compared by restriction enzyme analysis and pulsed-field gel electrophoresis (PFGE) (14). It is indicated that pyometra is caused by *E. coli* derived from the normal flora of the dogs and not by certain clones spread between animals (14). The DNA profiles of the isolates were 100% identical. This theory suggests that the urinary tract and uterus are infected with the same strain (14). In another study, isolates from bitches with pyometra and UTI were compared by biochemical phenotypes with isolates from the feces of healthy dogs. It was concluded that *E. coli* associated with canine pyometra derived from the fecal flora and that the urinary tract was infected by the same *E. coli* clone as the uterus of a bitch with pyometra (38). Again, *E. coli* strains associated with pyometra were characterized by biotyping and with plasmid profiles (13). Plasmid profiles of all plasmid containing isolates revealed plasmid bands corresponding to molecular weights ranging from 1 to 160 kb.

In the present study, EIEC was isolated from fecal samples (17%) and pyometra (5%) and hybridized with the *eae*A and VT1 probe. Similar to the present results, in a previous study in humans, 1 of the 11 EIEC strains and 1 of the 25 normal flora *E. coli* isolates hybridized with the *eae*A probe (24). However, it has been found that all major EIEC O serogroups produce low levels of verocell cytotoxins that are immunologically distinct from VT1 or VT2 of EHEC (O157) (39). In addition, EIEC DNA probes

have been reported to give occasional false positive results (i.e. there are strains that are probe positive but Sereny test negative), particularly when used to screen strains that have been stored for some time in the laboratory (40). At the same time, it is clear that bacteria that carry the invasive gene (and hybridize positively with the DNA probes) can lose their Sereny positivity. Presumably this is due to a spontaneous loss of critical plasmid or chromosomal gene sequences (41). However, similar to our findings, DNA hybridization studies suggest that many verotoxin-producing *E. coli* isolates from humans and cattle possess a chromosomal gene called *E. coli* attaching and effacing (*eae*A) (42-46). In this study, *eae*A positive but VT1 negative strains were isolated. These strains do not harbor the genetic information necessary for verocytotoxin production. Similar strains have been previously isolated from humans (46-49) and, most commonly, from animals (44,50). One possible explanation is that these strains may have lost the virulence genes, and therefore have a reduced potential for virulence. However, with toxins in some EPEC strains, toxigenicity may not necessarily be correlated with pathogenicity (51). However, it is convenient to relate the isolated organism to a particular serotype, but that should not be the final criterion, as it is possible that this non-typable organism later may become a potential pathogen or *E. coli*, which cause disease in dogs and may not limited by serotypes.

Previously, Fox and Haynes, (16) and later Wilkonson (18), discussed the possibility that the cause of uterine infection was *E. coli*. They serotyped the strains of *E. coli* from both rectal and pyometra samples. Serotyped *E. coli* strains were found with equal frequency in uterine and alimentary tract systems. Therefore, the gut might be the source of genital tract infection in dogs. Later, Sager and Remmers (52) carried out bacteriological cultures on 118 puppies that had died over an unstated period of time at an intensively operated dog breeding kennel. Bacterial septicemia occurred in 74% of the cases and beta-hemolytic *E. coli* was one of the more commonly isolated organisms. They concluded that infections arose from contamination in the uterus, during passage through the birth canal or from the mastitic milk of bitches. Wadas et al., (38) compared biochemical fingerprintings of fecal *E. coli* with *E. coli* from pyometra cases. They showed that in all 10 cases uterine *E. coli* isolates were identical or very similar to the isolates from the feces of the same bitch.

In the present study, CNF-positive strains were isolated from fecal and pyometra samples in dogs. Previously, CNF-positive strains were reported in fecal samples from dogs (53). However, CNF-positive strains were also isolated from the feces of a representative percentage of healthy children, cats and calves (54,55) and could belong to different serogroups and serotypes (9,56,57). The role of CNF strains as a cause of pyometra is unknown but CNF-positive *E. coli* strains have been reported to be associated with diarrhea and urinary tract infections in dogs (58).

These findings suggest that pathogenic *E. coli* may play an important role in pyometra and the strains isolated from the uteri may be similar to isolates of feces. However, the number of strains examined thus far is not sufficient to draw firm conclusions about the epidemiology of pathogenic *E. coli* in dogs with pyometra. Further studies are needed to examine their potential role in the pathogenesis of pyometra in dogs.

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