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 (*eaeA*), 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 *eaeA* (47%), *VT1* (29%) and *EIEC* (17%) compared with the uterine samples in which both *eaeA* 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 Düşkü ve Pyometra Örneklerinden İzole Edilen *Escherichia coli*’lerin DNA Hibridizasyonu İle İncelenmesi**

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

Düşkü ve pyometra örnekleri 17 dişi köpekden toplandı. *E. coli*bütün düşkü ve pyometra örneklerinden izole edildi. Her örnekten alınan *E. coli’lerin patojen özelliklerini belirlemek için HRP ile bağlanmış enteropatojenik (eaeA), verotoksik (VT1, VT2), enterohemorajik (EHEC), enterotoxik (ETEC), enteroaggregatif (EAggEC) ve enteroinvasiv (EIEC) DNA probing ile hibridizasyonlar yapıldı. Vero hücrelerinde de “Cytotoxic necrotizing factor” (CNF) özelliğini belirlemek için bir test uygulandı.


Bulgular patojenik *E. coli’nin köpeklerde pyometra oluşumunda önemli bir rol oynayabileceği ve köpeğin kendi dışkılarından da köken alabileceği 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 (CNF1 and CNF2), 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, Basingstoke, 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’Étoile, 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 (eaeA), 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,
Amersham, RPN 3001) according to the manufacturer’s instructions.

**Cytotoxicity 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 *eaeA* ($n = 8, 47\%$), VT1 ($n = 5, 29\%$) and EIEC ($n = 3, 17\%$) compared with the uterine samples in which both *eaeA* 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 *eaeA* gene.

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

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**Table 1. Derivation of the 7 gene probes.**

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Plasmid</th>
<th>Endonuclease</th>
<th>Fragment (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETEC (LTh, ST1a, ST1b)</td>
<td>pKAD008</td>
<td>$Xba\ I$</td>
<td>1268</td>
<td>23</td>
</tr>
<tr>
<td><em>EaeA</em></td>
<td>pCVD434</td>
<td>$Sal\ I + Kpn \ I$</td>
<td>1000</td>
<td>24</td>
</tr>
<tr>
<td>EIEC</td>
<td>H1(60R706)</td>
<td>$EcoR\ I + Sal\ I$</td>
<td>1000</td>
<td>25</td>
</tr>
<tr>
<td>EHEC</td>
<td>pCVD419</td>
<td>$Hind\ III$</td>
<td>3400</td>
<td>26</td>
</tr>
<tr>
<td>VT2</td>
<td>pACYC184</td>
<td>$Sma\ I + Pst\ I$</td>
<td>850</td>
<td>27</td>
</tr>
<tr>
<td>EAggEC</td>
<td>pCVD432</td>
<td>$EcoR\ I + Pst\ I$</td>
<td>700</td>
<td>28</td>
</tr>
<tr>
<td>VT1</td>
<td>pACYC177</td>
<td>$Hinc\ II$</td>
<td>750</td>
<td>29</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Class</th>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAggEC</td>
<td>Wilmott</td>
<td>human EAggEC strain</td>
<td>J. W. S*</td>
</tr>
<tr>
<td>Lewis</td>
<td>human EAggEC strain</td>
<td>J. W. S*</td>
<td></td>
</tr>
<tr>
<td>Hoque</td>
<td>human EAggEC strain</td>
<td>J. W. S*</td>
<td></td>
</tr>
<tr>
<td>17-Sub</td>
<td>from a child with diarrhea in Santiago, Chile</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>VTEC</td>
<td>E30480</td>
<td>serotype O157:H7, contains VT1, VT2, <em>eaeA</em></td>
<td>30</td>
</tr>
<tr>
<td>E32511</td>
<td>serotype O157:H- contains VT2 and <em>eaeA</em></td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>ETEC</td>
<td>E5798</td>
<td>serotype O7:H18, contains LT*</td>
<td>31</td>
</tr>
<tr>
<td>E2965</td>
<td>serotype O159:H34, contains ST*, LT*</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>E7476</td>
<td>O166:H27, contains ST*</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>B44</td>
<td>serotype O9:K30:K99, contains ST*</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>EPEC</td>
<td>E2348/69</td>
<td>serotype O127:H6 implicated in an outbreak of infant diarrhea in England</td>
<td>33</td>
</tr>
<tr>
<td>RDEC-1</td>
<td>serotype O15:NM, rabbit strain</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>EIEC</td>
<td>EI314</td>
<td>serogroup O124</td>
<td>35</td>
</tr>
</tbody>
</table>

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

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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 eaeA 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 eaeA 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 eaeA 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 (eaeA) (42-46). In this study, eaeA 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.

Acknowledgments

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References


