O Mundo da Saúde, São Paulo - 2017;41(3):378-384
Artigo Original • Original Paper

Determining the pathogenicity of bacteria present in wetland waters and crow feces in Bothell

Determinando a patogenicidade de bactérias presentes nas águas dos pântanos e em fezes de corvos em Bothell

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Abstract

This study will determine the pathogenicity of bacteria isolated from the wetland waters and crows feces within the UW Bothell campus. This emanated from the need to determine whether the American crow (*Corvus brachyrhyncos*) has a role in the epidemiology of diarrheal disease along with its significant con-tribution to the high level of fecal coliforms in the stream water that runs through the crow roosting area. Modified from previous studies, we developed protocols to culture *Escherichia coli* and *Campylobacter*, both are known to be pathogenic and present in crow feces, to isolate DNA from cultures or samples, and to perform PCR (Polymerase Chain Reaction)/qPCR (Quantitative Polymerase Chain Reaction) to detect virulence genes. We found that the virulence genes *eae* and *rfb* that are necessary to cause diarrhea were absent in a representative number of E. coli strains isolated from the water samples and the fecal samples. The virulence genes *flaA* and *cad* in the *Campylobacter* species were detected in fecal samples (77.8% and 73%, respectively) and in water samples (75% each). In conclusion, our hypothesis could not be verified, but our results suggest that the *Campylobacter* isolated from wetland water and crow feces are potentially pathogenic. However, the results are not conclusive and more sample and virulence genes specific to ex-traintestinal pathogenic E. *coli*, need to be screened in order to accurately assess the pathogenicity of these bacteria.

Keywords: Escherichia coli. Campylobacter. Virulence genes.

Resumo

Este estudo determinará a patogenicidade de bactérias isoladas das águas pantanais e fezes de corvos den-tro do campus UW (University of Washington) Bothell. Este estudo emanou da necessidade de determinar se o corvo americano (Corvus brachyrhyncos) tem um papel na epidemiologia da doença diarréica, junta-mente com sua contribuição significativa para o alto nível de coliformes fecais na água do fluxo que per-corre a área onde as aves se alojam. Modificado a partir de estudos anteriores, desenvolvemos protocolos para cultura de Escherichia coli e Campylobacter, ambas conhecidas por serem patogênicas e presentes em fezes de corvos, isolar DNA de culturas ou amostras e realizar PCR/qPCR para detectar genes de virulên-cia. Descobriu-se que os genes de virulência *eae* e *rfb* que são necessários para causar diarreia estavam au-sentes num número representativo de estirpes de E. coli isoladas das amostras de água e das amostras fe-cais. Os genes de virulência *flaA* e *cad* em espécies de Campylobacter foram detectados em amostras fecais (77,8% e 73%, respectivamente) e em amostras de água (75% cada). Concluindo, nossa hipotese não pôde ser confirmada, mas nossos resultados sugerem que a Campylobacter isolada das amostras de água e de fezes de corvo é potencialmente patogênica. No entanto, os resultados não são conclusivos e mais amostras e genes de virulência específicos para E. coli extraintestinal patogênica necessitam ser identificados, a fim de avaliar com precisão a patogenicidade destas bactérias.

Palavras-chave: Escherichia coli. Campylobacter. Genes de virulência.

DOI: 10.15343/0104-7809.20174103378384

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INTRODUCTION

There has been a growing concern for the water quality in the wetland stream that runs through the UWB (University of Washington Bothell) campus. In 2012, water samples upstream of the campus taken from North Creek were collected and analyzed, revealing that the wetland depressions in North Creek had remarkably high levels of fecal coliform bacteria[1]. Interestingly, a timescale analysis of the wetland water showed that the levels of fecal coliform bacteria counts appear to subside in the spring and summer, yet increase during the fall and winter[2].

Researchers infer that the fluctuation may be a result of the change in fecal load associated with seasonal migration of the American crow. Over the past years, crows gathering on campus during the even-ing has been a mesmerizing sight as thousands gather to roost in the wetland restoration area. Varying from a few thousand in the spring-summer months to as many as 15,000 crows during the fall-winter months, these crows accumulate a massive deposit of nitrogenous and fecal waste[2]. A study showed that E. *coli* bacteria counts in a few surface water samples collected within the roosting area of the wetland have been in the 10,000-100,000 CFU (Colony Forming Units)/100ml range, which is supportive of the claim that the influx of crows during winter roosting season contributes an excess of fecal coliform bacteria from their droppings[3]. This raises a concern for whether these coliform bacteria can have a health im-pact on local inhabitants, along with their apparent impact on the local environment.

No studies have been done to determine whether these bacteria possess virulence genes that can al-low them to invade and colonize a human host, making them potentially pathogenic. Therefore, the aim of this study is to determine the pathogenic potential of the bacteria isolated from the water samples and crow feces.

From previous studies, E. *coli* and *Campylobacter* spp. are known to be the leading cause of human bacterial diarrheal disease in both developed and developing countries [4,5,6]. Furthermore, E. *coli* and *Cam-pylobacter* spp. have also been isolated from crows and other avian reservoirs in previous studies [7,8,9,10,11]. Therefore, these bacteria will be our species of interest in determining the pathogenic potential, which will be isolated from the water samples and crow feces in the wetland restoration area.

We expect to find both E. *coli* and *Campylobacter* species from the wetland water samples and the crow feces. Once we have confirmed the presence of these species we will screen for virulence genes re-spective to each species to determine their pathogenicity. The virulent genes to be screened in E. *coli* are *sfaS, fyuA, eae*, and rfb. The virulent genes to be screened in *Campylobacter* species are *Camp2*, *flaA*, and *cadF*. We hypothesized that over 50.00 % of the samples of E. *coli* and the *Campylobacter* species col-lected from crow feces would contain at least one of our selected virulent genes.

MATERIALS E METHODS

We searched in the literature to find virulence genes, PCR protocols, and various culturing tech-niques for E. coli and Campylobacter spp. [12,13,14].

Water and Feces collection and preparation

From May 2014 to January 2015, Dr. Rob Turner's group, from Department of environmental sci-ence, collected water samples (n=93) and fecal samples (n=40) from the surrounding UWB wetland (Fig-ure 1). The water samples included Site 3 (n=9), Site 4a (n=15), SW (Surface Water) (n=21), NC (North Creek) (n=22), RP (Runoff Pond) (n=2), SR (Sammamish River) (n=14), and RS (Runoff Swale) (n=10). The water samples were diluted to various concentrations from 1.0%-50.0%, to be membrane filtrated (Hach) and grown on m-ColiBlue 24 filters (Hach) that allow detection of E. coli species as blue colonies. The feces were collected from different crow droppings using sterile swabs. About 1g of feces sample was suspended in 500 µL PBS (Phosphate Buffered Saline) buffer in microfuge tubes and processed immedi-ately as described below.

Isolation of Escherichia coli

Feces samples and representative E. coli colonies on m-Coliblue filters were cultured in EMB (Eo-sin Methylene Blue) media. EMB media is selective for gram-negative bacteria and differential for E. coli, forming an exuberant green metallic sheen[15]. Each EMB plate was divided into four or eight sections with an isolated colony placed on each section. The plates were incubated at 37° C for 24-48 hours.

Isolation of Campylobacter

Campylobacter spp. are microaerophilic bacteria. Hence, prolonged exposure to oxygenic environ-ment will leave less viable cells, so exposure of fecal samples to oxygen should be minimized[16 17]. 200 µL of feces samples were pipetted onto blood agar through a membrane filter paper so that only the *Campylo-bacter* would pass, leaving the debris atop. The blood agar was incubated at 37° C for 1 hour, then the filter was removed. 10 µL of the feces sample, were pipetted onto Karmali agar and streaked evenly. Both blood agar and Karmali agar cultures were incubated at 37° C under microaerophilic conditions in Campy Pouch (Camp Gen inc.) for 48-72 hours. Karmali agar is a selective medium for the gram-negative *Cam-pylobacter jejuni* and coli when incubated at 42° C[18]. Blood agar is a differential medium discerning whether the bacteria has hemolysin, a trait common in pathogenic bacteria and which changes the color of the media when present[19].

DNA Extraction from filters and feces

Two different protocols were used for DNA extraction. In one method, total DNA was extracted directly from the filters or the fecal samples skipping the process of culturing/ isolation before obtaining pure DNA for PCR/ qPCR run. All DNA material present was extracted regardless of their origin. The WaterMaster™ DNA purification Kit (Epicentre biotechnologies) was used, and 600 µL of 1x Lysis Solution were added onto the filter and gently pipetted five times. The filtrate was transferred into a microfuge tube. 2 µL of Proteinase K (50 µg/ µL), were added into the tube and centrifuged at 14,000x g for 10 seconds to disperse foam. The tube was incubated in a 65-70° C water bath for 12 minutes. The tube was cooled to 37° C. 2 µL of RNase A (5 µg/ µL) were added and vortexed for 5 seconds. The tube was incu-bated at 37° C for 30 minutes. The tube was cooled on ice for 3-5 minutes. MPC protein precipitation solu-tion (350 µL) was added to the tube and vortexed for 10 seconds. The tube was centrifuged for 10 minutes to pellet the debris. The supernatant was transferred into a 2 mL microfuge tube.

The isopropanol was carefully removed leaving the DNA pellet. The DNA pellet was rinsed with 70% ethanol and it was carefully removed from the DNA tube. The DNA pellet was air dried then resuspended in 50 µL DNase-free water.

DNA extraction from pure cultures and stock freezer preservation

The second method used the Prepman Ultra Sample Preparation Reagent kit (Life Technologies) to extract the DNA directly from a bacterial culture. E. coli colonies were inoculated into 1mL TSY (Tryptic Soy Yeast) broth in a microfuge tube and incubated at 37° C overnight. Turbidity in the tube indicated growth. The culture, of 200 µL, was transferred into another microfuge tube. 200 µL of 80% glycerol was added into the remaining 800 µL of broth to reach a final concentration of 20% glycerol. The tube was inverted to mix, then stored in a -80° C freezer as stock cultures. The tube of 200 µL cul-ture was centrifuged at 14,000x g for 1 minute to get a pellet. The supernatant was removed leaving the pellet behind. Prepman Reagent (50 µL) was added into the tube with the pellet and it was resuspend-ed by vigorous vortexing.

The tube was placed in a 95° C water bath for 10 minutes, cooled to room temperature, and centrifuged at 10,000x g for 2 min. Colonies were also picked from TSY plates (E. coli) or blood agar plates (Campylobacter) and directly resuspended into 50 µL of Prepman reagent. The suspension was heated as above and 2 µL of the cooled supernatant were used in PCR or qPCR.

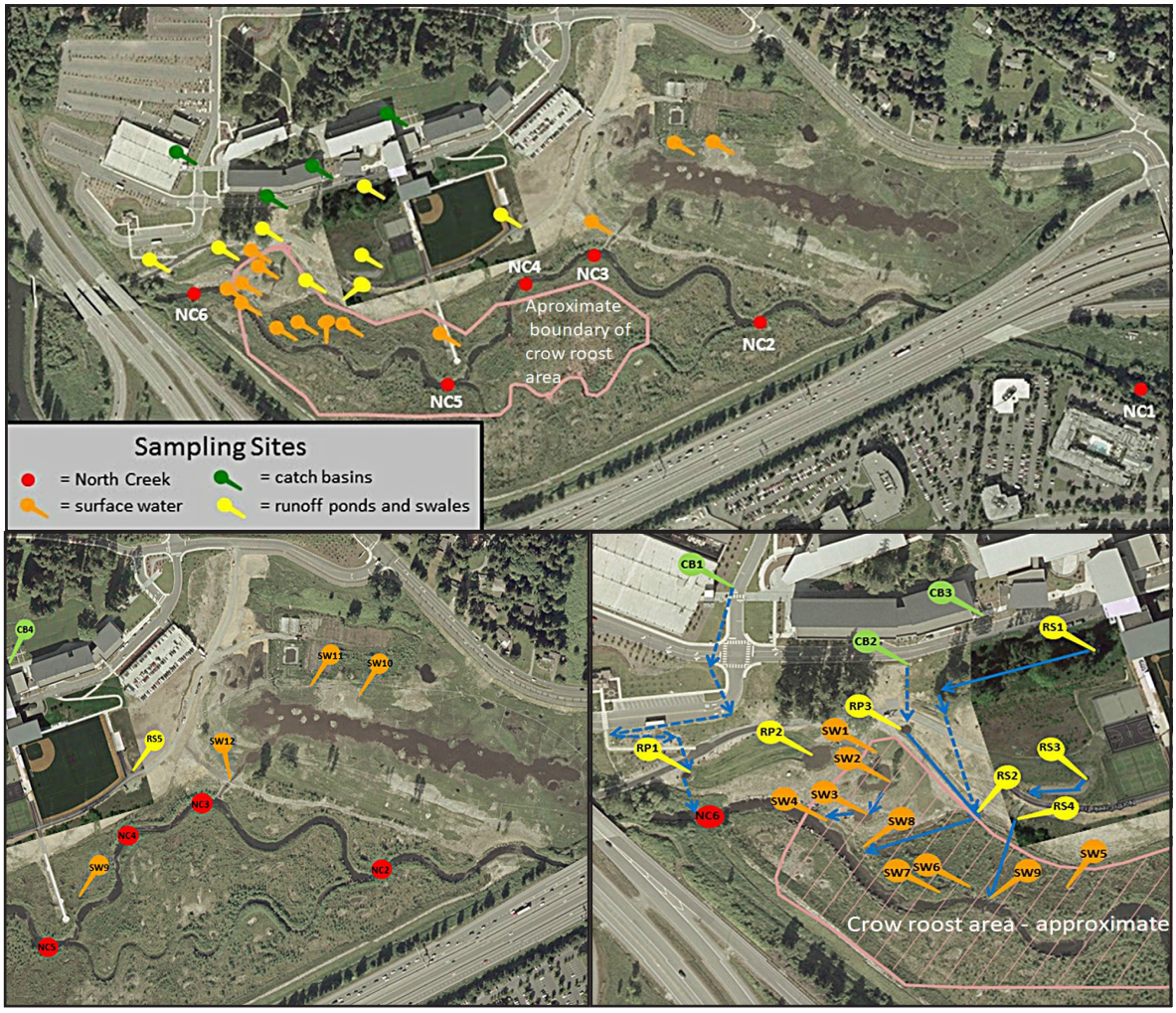

Figure 1 – Wetlands Sampling Sites. Source: Image provided by Phillip Van Valkenburg, 2013. Personal Colletion.

Virulence Genes

To identify the virulence genes we used the PCR and qPCR methods. S-fimbriae (sfaS), Yersiniabactin (fyuA), and E. coli O157:H7 marker (rfb and eae) are markers of pathogenic E. coli 20, 21.

To identify these markers, the methods chosen utilizes similar pairs of primers (forward and reverse), which are small sequences of RNA or DNA used by enzymes in the reaction to start the DNA replication, amplifying the desired markers. The sequences are described as letters that represent the nucleotides for a better understanding. The forward (F) and reverse (R) primers for the virulence genes are described in the table below. In addition, we used probes (a luminescent marker used in qPCR) for the eae gene (5TGGACATAGCATCAGCATA3'MGBNFQ) and for the rfb gene (5'VICTGVGGAACAAAACC3'MGBNFQ).

For *Campylobacter* species, we used primers for *Camp2* [22], flaA [23, 24] , and cadF 25genes, and a probe as well for *Camp2*(5'-FAMCAGAGAAC AATCCGAACTGGGACABHQ1-3').

The Flagellin A is necessary for human pathology for Campylobacter 14. The cadF gene is neces-sary for attachment of *Campylobacter* in the human intestine and is required for human pathology.

All primers were made by SIGMA-Aldrich and probes by Life Technologies.

PCR and Gel-Electrophoresis

PCR protocols were adapted from previous

studies on *Campylobacter* [14]. Amplification was done in a 25 µL reaction mixture that included: 12.5 µL of Jumpstart™ REDTaq® ReadyMix™ (SIG-MA); 2 µL of flaA primers containing 0.2 µM of each primer; 3 µL of DNA extract; and all were dilut-ed with nuclease-free water to a final volume of 25 µL.

Amplification was done in a BioRad thermal cycler with the protocol optimized for flaA: 95° C for 5 min, then followed by 35 cycles of 95° C for 1 min, 50 °C for 1 min, and 72° C for 90 seconds, then final extension at 72° C for 10 sec. PCR condi-tions for E. coli genes sfaS and fyuA were as described by Johnson et al [12]. The amplicons were separat-ed on a 0.6%-1% agarose gel by electrophoresis at constant voltage of 100 volts, and compared against a low molecular ladder after staining with SYBR safe dye.

qPCR

qPCR was carried out in a 20 µL reaction mixture. The final concentrations of the primers and probes for the gene targets eae and rfb were 0.3 µM and 100 nM, respectively. For the DNA template, 2 µL from a Prepman exrtraction of a 1 mL culture was used. For *Campylobacter* spp. specific qPCR, primer and probe concentrations and conditions were as described by Lund et al [22]. qPCR was performed in a MiniOpticon real-time PCR detection system.

Table 1 – E. coli. primers

Gene	Primer Sequence
sfaS	F 5´ - GTGGATACGACGATTACTGTG - 3´ R 5´ CCGCCAGCATTCCTGATTC - 3´
fyuA	F 5 ´ TGATTAACCCCGCGACGGGAA - 3´ R 5´ CGCAGTAGGCACGATGTTGTA - 3´
eae	F 5´ GTAAGTTACACTAAAAGCACCGTCG - 3´ R 5 ´ TCTGTGGATGGTAATAAATTTTTG - 3´
rfb	F 5´ TCAAAAGGAAACTATATTCAGAAGTTTGA - 3´ R 5 ´ CGATATACCTAACGCTAAAGCTAA - 3´

Table 2 – *Compylobacter* primer

Gene	Primer Sequence
camp2	F 5´ - CACGTG CTA CAA TGG CAT AT - 3´ R 5´ GGC TTC ATG CTC TCG AGT T - 3´
flaA	F 5 ´ GGATTTCGTATTAACACAAATGGTGC - 3´ R 5´ CTGTAGTAATCT TAAAACATTTTG - 3´
cadF	F 5´ TTGAAGGTAATTTAGATATG - 3´ R 5 ´ CTAATACCTAAAGTTGAAAC - 3´

RESULTS

E. coli from water samples

We isolated a total of 72 blue colonies from 93 water samples, and successfully plated them on EMB media. 68/72 (94.4%) of the colonies in EMB plates developed GMS (Green Metallic Sheen) in-dicative of the presence of lactose-fermenting-gram-negative *E. coli*.

PCR and gel electrophoresis of those E. coli selected from SW2 (n=7), SW9 (n=1), SW12

(n=1), NC1 (n=1), NC5 (n=13), and NC6 (n=1) all gave negative result for the virulent genes *eae* and *rfb*. Zero were positive out of the 24 samples screened (0/24, 0%).

Campylobacter from water samples

We performed DNA extraction on isolates from 5 water sample sites. NC1 (n=1) and NC6 (n=1) yielded negative results, while NC5 (n=1), SW2 (n=1), and RP3 (n=1) gave positive results for the *Campylobacter* marker.

E. coli from fecal samples

We isolated a total of 130 colonies from 40 fecal samples and plated them on EMB media. 123/130 (94.6%) of the colonies developed GMS indicative of the presence of *E. coli*.

PCR and gel electrophoresis of those E. coli selected from F1:1, F2:1, F3:2, F4:1, F5:1, F6:1, F7:1, F8:1, and F9:1 all gave negative results for the virulence genes *sfa* and *fyu*. Zero were positive out of the 9 samples screened.

Campylobacter from fecal samples

Out of the 20 fecal samples that we plated on Karmali agar, 14 showed growth of *Campylobac-ter* (14/20, 70%) as indicated by tiny-white colonies. Out of the same 20 fecal samples that we plated on blood agar, 6 showed growth of microaerophilic bacteria as tiny translucent colonies (6/20, 30%). Phase-contrast microscopy was used to verify that the isolates were *Campylobacter* by their characteris-tic spiral shape.

qPCR of both DNA prep and DNA extract showed that 67.5% of fecal samples were positive for the *Campylobacter* marker. And by PCR and gel electrophoresis: 77.8% and 73% of those Campyl-obacter-marker-containing fecal samples were also positive for the virulence gene *flaA* and *cad*, respec-tively.

DISCUSSION

The aim of this study was to determine the pathogenic potential of bacteria isolated from water samples and crow fecal samples in the wetland restoration area. The result of our *E. coli* isolation demonstrated to be an effective method for detecting and culturing *E. coli* isolated from water sample (68/72, 94.4%) and fecal sample (123/130, 94.6%). The results of our PCR and gel electrophoresis sug-gest that E. coli isolated from water sample (0/24, 0%) and fecal sample (0/9, 0%) do not contain the eae, rfb, sfa, and fyu virulence genes. The result of our *Campylobacter* isolation revealed Karmali agar was more effective for detecting and culturing of *Campylobacter* (14/20, 70%), than on blood agar (6/20, 30%), as confirmed by qPCR analysis.

The results of qPCR, PCR/gel electrophoresis of DNA preparation from isolated colonies as well as total DNA extract suggest the presence of virulent Cam-pylobacter with the flaA gene in fecal samples (21/27, 77.8%) and water samples (3/5, 60%), and the cad gene in fecal samples (19/26, 73%). These methods can be further optimized for future studies on the microbes in wetland waters and crows' feces, which have been suggested from our results to be potentially pathogenic.

Our detection of *Campylobacter* and *E. coli* in the crows roosting around the wetland restora-tion area is consistent with similar studies conducted in other suburban areas of other countries, namely in Japan, New Zealand, Malaysia, and Tanzania [7,8,9]. In addition, we also detected virulent genes in Campylobacter which suggests that these bacteria are pathogenic and can cause diarrheal disease in humans. If it is true that the crows are the cause of the influx of fecal bacteria, then initiative action should be taken to ensure that students and local inhabitants are protected from zoonotic infection from crows.

CONCLUSION

In conclusion, our hypothesis that over 50% of the bacteria would present at least one of the virulence genes could not be confirmed. However, being an initial investigation with developing bacte-ria isolation techniques, genetic screening methods, and a relatively small sample size, our results should be taken

with caution.

These explanations may account for why we have not yet detected any virulence genes in *E. coli* isolated from both water samples and fecal samples. More samples and more virulence genes need to be screened in order to determine the full pathogenic potential of these bacteria.

ACKNOWLEDGEMENTS: We thank Dr. Keya Sen for help with the design and execution of experiments and guidance through-out the research. We thank Professors Turner, Servetnick, and Hillesland for the generous use of their water samples, lab workstation, and lab reagents, respectively. Many thanks also to Phillip Van Valken-burg and William Jonsson for sample collection, Christy Cherrier and Sujung Lim for technical support, and EPA for the positive control samples.

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Received in December, 2016.
Approved in July, 2017.