Development of a DNA probe colony hybridization hydrophobic grid membrane filter method for detection of *Escherichia coli* isolates carrying virulence and antimicrobial resistance genes in the pig intestinal microflora.

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Abstract
The objective of this study was to develop a DNA probe hybridization procedure using hydrophobic-grid membrane filters (HGMFs) for the detection of *E. coli* isolates carrying specific virulence or antimicrobial resistance genes. Genes were detected using digoxigenin-labelled probes and an alkaline-phosphatase substrate. Specifically, the *elt*, *esiB*, and *fesG* genes encoding the enterotoxins LT, ST, and fimbrial adhesin F4 were targeted. The hybridization procedure was optimized using *E. coli* strain ECL7805 which possesses the three targeted genes, and rectal swabs from weaned pigs excreting *E. coli* positive for these genes, as demonstrated by enrichment PCR. The preliminary results of this study suggest that this method is highly sensitive, specific, and easily accessible for any laboratory. This method is also convenient for screening for virulence genes present in low numbers in feces of pigs since we can target about 200 colonies simultaneously. This approach will permit the study of the dynamics of virulence and antimicrobial resistance genes in the intestinal *E. coli* population of swine and other species.

Introduction
*Escherichia coli* represent a dominant part of the non-anaerobic intestinal bacterial population in swine. They include a broad variety of different clones, ranging from highly pathogenic strains causing severe disease in the swine population to non-pathogenic commensal *E. coli* (3, 5, 8). Pathogenicity is characterized by the presence of certain combinations of specific virulence factors, and diseases caused by pathogenic *E. coli* are responsible for major economic losses. In addition, shiga toxin-producing *E. coli* O157:H7, an important cause of food born illness in humans, has been recovered from feces and carcasses of healthy pigs (3, 8), and cases of human illness have been traced back to pig products. In addition, a high prevalence of antimicrobial resistance genes in generic *E. coli* has been found in production pigs in several studies (2, 3). *E coli* are also considered as an important source of antimicrobial resistance determinants for other pathogens (7). Thus, the detection and monitoring of virulence and antimicrobial genes in the pig intestinal *E coli* population is very important.

PCR and colony hybridization with radioactively labeled probes are both routinely used in detection of virulent *E. coli*. Although PCR methods can detect the presence of low numbers of virulence and antimicrobial resistance genes in mixed cultures, confirmation of virulence and antimicrobial profiles of individual strains in the population is arduous and time-consuming. The DNA probe hybridization procedure using hydrophobic-grid membrane filters (HGMFs) is an alternative to the traditional methods for the detection of *E. coli* colonies carrying specific genes. The overall objective of this research is the monitoring and control of virulent and antimicrobial resistant *E. coli* strains, particularly those associated with food born infections, among populations of swine and other species. In the initial phase of this work, we examined pure cultures of *E. coli*, and rectal swabs from weaned pigs, for the detection of a limited number of virulence genes. The goal of this step was to evaluate the sensitivity and specificity of the DNA probe hybridization HGMF method and to evaluate the ability of this method to identify multiple colony virotypes in any one sample. This will permit the sampling and screening of a larger number of virulence and resistance genes in order to monitor the dynamics of virulence and antimicrobial resistance types in an *E coli* population.

Materials and methods

*Safe pork 2009 - Québec city, Québec, Canada*
Bacterial strains
The wildtype E. coli O149 strain ECL7805 which possesses the elt, estB, and faeG genes encoding the enterotoxins LT, STb, and fimbrial adhesin F4 respectively was used as a positive control. The wild-type non-pathogenic O115: K- strain ECL3463 which does not possess any of the target genes was used as a negative control. Strains were provided by the E. coli Laboratory (EcL) of the Faculté de Médecine Vétérinaire of the Université de Montréal.

Samples
Three rectal swabs taken from different clinically healthy pigs from the same group following weaning were tested positive for LT, STb, and F4 by PCR following overnight enrichment in Luria Bertoni (LB) broth. Cultures were stored at -20°C in 25% glycerol for the determination of colony virotypes.

Filtration and replication
Strains ECL7805 and ECL3463 were incubated overnight in tryptic soy broth (TSB) and serially diluted to 10⁷ in TSB. A volume of 2 ml of this dilution was mixed with 3 ml of 1% tween 80 solution, and filtered through a HGMF using the spreadfilter (Filtrflex, Almonte, Ontario, Canada). HGMFs were placed onto MacConkey agar plates and incubated overnight at 37°C. HGMFs showing about 200 bacterial colonies were considered as masters and were replicated using a microbial colony replicator (Filtrflex, Almonte, Ontario, Canada), placed onto MacConkey agar plates and incubated overnight at 37°C. LB cultures from the rectal swabs were serially diluted in TSB to 10⁷, filtered through HGMFs and replicated as described above.

Preparation of DNA probes
The DNA templates were prepared from an overnight LB culture of ECL7805 strain and were used as probes for the detection of the elt, estB, and faeG genes. Digoxigenin (DIG) alkaline phosphatase labeled probes were generated using specific primers (Table 1). The DNA templates were labeled according to DIG labeling kit (Roche Diagnostics) instructions.

Pretreatment, hybridization, and colorimetric detection
HGMF copies were pretreated using pretreatment solution (45 ml of 10 mM Na2PO4 [pH 6.0], 9 ml of 1M NaHCO3, 135 ml of polyethyleneimine), lysis solution (150 mM NaOH in 70% ethanol), proteinase K solution, rinsing solutions, and UV in order to lyse and fix the colonies on the grid cell before prehybridization, as described previously (10). The prehybridization and hybridization steps were carried out at 68°C in 6 X SSC (20 X SSC is 0.3M sodium citrate plus 3 M sodium chloride) containing 0.5% SDS, 1% of blocking reagent and 100μg DNA of salmon sperm per ml. In addition, the hybridization solution contained 15 ng/ml of denatured specific probe. The presence of specific genes was revealed colorimetrically with the alkaline phosphatase substrate, 5-bromo-chloro-3-indolyl phosphate (BCIP), and nitroblue tetrazolium salt (NBT) which react after the dephosphorylation, showing a dark blue indigo dye as an oxidation product, according to instructions for the DIG Nucleic Acid Detection Kit (Roche Diagnostics).

Results
About 100 to 200 colonies were isolated on HGMFs following filtration of the 10⁷ dilution of the pure cultures and rectal swab enrichment cultures, and these colonies were considered as single CFU colonies (9). From rectal swabs, at least 3 types of colony grew on the HGMFs. The predominant colony type was intensely red and each colony filled an HGMF grid cell. The type of colony is identical to that observed after the filtration of E. coli pure cultures, as shown in Figure 1. These colonies were considered as presumptive E. coli colonies.
All 9 replicate HGMFs from the ECL7805 strain filtration were positive for LT, STb, and F4, by the DNA probe colony hybridization HGMF method, colonies demonstrating a dark-blue indigo coloration (Figure 2). Almost 100% of the colonies of the every replicate HGMF were positive suggesting a very high sensitivity of the method. All colonies from 9 replicate HGMFs obtained by filtration of strain ECL3463
were negative for LT, STb, and F4 by DNA probe colony hybridization, suggesting also that the method is highly specific.

The three LB broth enriched rectal samples which were positive for LT, STb, and F4 by PCR demonstrated positive colonies for one or more of these factors on the DNA probe colony hybridization HGMF method (Table 2). One sample demonstrated a mix of 2 virotypes, LT:STb:F4 and STb, whereas the two other samples demonstrated only the STb virotype. In all cases we did not find more than 5 positive colonies among 100 or more colonies that grew on the same HGMF.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Primer sequence</th>
<th>Tm</th>
<th>size</th>
<th>Control strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>STb</td>
<td>for 5' GCA ATA AGG TTG AGG TGA T</td>
<td>60°</td>
<td>368</td>
<td>ECL 7805</td>
</tr>
<tr>
<td>STb</td>
<td>rev 5' GCC TGC AGT GAG AAA TGG AC</td>
<td>60°</td>
<td>368</td>
<td>ECL 7805</td>
</tr>
<tr>
<td>LT</td>
<td>for 5' TTA CGG CGT TAC TAT CCT CTC TA</td>
<td>60°</td>
<td>275</td>
<td>ECL 7805</td>
</tr>
<tr>
<td>LT</td>
<td>rev 5' GGT CTC GGT CAG ATA TGT GAT TC</td>
<td>60°</td>
<td>275</td>
<td>ECL 7805</td>
</tr>
<tr>
<td>F4(K88)</td>
<td>for 5’ ATC GGT GGT AGT ATC ACT GC</td>
<td>60°</td>
<td>601</td>
<td>ECL 7805</td>
</tr>
<tr>
<td>F4(K88)</td>
<td>rev 5’ AAC CTG CGA CGT CAA CAA GA</td>
<td>60°</td>
<td>601</td>
<td>ECL 7805</td>
</tr>
</tbody>
</table>

Table 2: Detection of multiple *E. coli* virotypes in the feces of normal weaned pigs by the DNA probe colony hybridization hydrophobic grid membrane filter method

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genes detected in the sample</th>
<th>Colony virotype (No of positive colonies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>STb, LT, F4</td>
<td>LT:STb:F4 (2); STb (3)</td>
</tr>
<tr>
<td>2</td>
<td>STb, LT, F4</td>
<td>STb (3)</td>
</tr>
<tr>
<td>3</td>
<td>STb, LT, F4</td>
<td>STb (3)</td>
</tr>
</tbody>
</table>

1  Fecal swabs were enriched in LB broth and tested by PCR for LT, STb, and F4

**Figure 1:** *E. coli* colonies on hydrophobic grid membrane filters after overnight incubation of a pure culture of strain ECL7805 on McConkey agar at 37°C

**Figure 2:** LT positive colonies on a hydrophobic grid membrane filter after overnight incubation of a pure culture of strain ECL7805 on McConkey agar at 37°C, as detected by DNA probe colony

**Discussion**

Our results following examination of the strains ECL7805 and ECL3463 known to be positive and negative respectively for the *elt*, *estB*, and *faeG* genes confirm the high sensitivity and specificity of the DNA probe colony hybridization HGMF method, as has been reported by Yan et al (11). The detection limit was been measured but we consider that this method is interesting compared to the traditional method where a limited number of colonies are randomly selected for the detection of the genes, since we can target about 200 colonies simultaneously. Todd et al. (10) reported a limit of 0.1 CFU/ g for the detection of shiga toxin-producing *E. coli* in artificially inoculated ground beef. Assuming a growth rate equivalence in the *E. coli* population during enrichment and as we found a low number of positive colonies, this suggests that colonies carrying LT, STb, and F4 are present in very low numbers in the feces of healthy pigs, despite the PCR positive test. Indeed, LT:STb:F4 isolates are considered to be causative
agents of diarrhea in postweaning pigs, and are rarely present in the intestinal microflora of clinically normal animals (4). Nevertheless, in spite of the low numbers of positive colonies, we were able to distinguish a mixed population containing more than one virottype. These preliminary results provide encouraging evidence that the DNA probe colony hybridization HGMF method will be very useful for the study of the effect of various interventions on the dynamics of virotypes and antimicrobial resistance profiles which are present in the intestinal microflora in greater numbers.

Conclusion
The DNA probe colony hybridization HGMF method permits the identification of the presence of gene combinations in E. coli isolates and provides an accessible means to follow the dynamic of virulence and antimicrobial resistance types in an E.coli population.

References