Consumption of Antimicrobial-Resistant *Escherichia Coli*-Contaminated Well Water: Human Health Impact

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M. Salvadori
B.L. Coleman
M. Louie
S. McEwen
A. McGeer

STATEMENT OF OBJECTIVES

The use of antimicrobial agents in agriculture, food production, and health care impacts human health through the natural selection and emergence of antimicrobial resistance in many strains of bacteria. Antimicrobial resistant organisms can evolve due to antimicrobial pressure directly on host bacteria (e.g. in the large bowel of humans treated with antibiotics), or from selection pressures imposed on free-living microbial communities due to the presence of antimicrobial residues. Environmental contamination, including contamination of water, with antimicrobial resistant organisms can occur as a result. Inadvertent ingestion of the contaminated material, food, and/or water can lead to gastrointestinal colonization. The human gastrointestinal system can become colonized with many different strains of bacteria, including *Escherichia coli (E. coli)*. Antimicrobial resistance in *E. coli* is of particular concern because it is the most common Gram-negative pathogen in humans, the most common cause of urinary tract infections, and a common cause of both community- and hospital-acquired bacteraemia. In addition, resistant *E. coli* strains have the ability to transfer antibiotic resistance determinants not only to other strains of *E. coli,* but also to other bacteria within the gastrointestinal tract.

We hypothesize that (1) people who consume water contaminated with antimicrobial resistant *E. coli* are more likely to harbour antimicrobial resistant *E. coli* in their gastrointestinal tract than people who consume uncontaminated water and (2) that this colonization will persist over time. We propose to:

i) identify risk factors for private well water contamination with antimicrobial resistant *E. coli*
ii) measure faecal carriage of antimicrobial resistant strain(s) of *E. coli* in household members who consume private well water
iii) determine associated risk factors for faecal carriage of an antimicrobial resistant strain(s) of *E. coli*
iv) measure the change in faecal carriage of antimicrobial resistant strain(s) of *E. coli* at baseline and three months
v) determine factors associated with changes in faecal carriage of an antimicrobial resistant strain(s) of *E. coli* from baseline to three months.

BACKGROUND

The problem of antimicrobial resistance has increased rapidly in the last decade and is a major public health threat worldwide (Koplan, 2000). A report completed by the Canadian Committee on Antimicrobial Resistance (2002) estimates that resistant organisms add at least $14.2 to $25.5 million per year to the direct cost of treating infections in Canadians. Additional costs are incurred for increased morbidity and mortality, and in screening and isolating people found to be carriers of antimicrobial resistant bacteria. The greatest concern is the potential loss of effective therapy for infections associated with antimicrobial resistant bacteria. The inability of pharmaceutical research to identify and develop any new antimicrobial classes in the last 15 years obviously adds to the level of concern.

The role of human antimicrobial use in selecting for resistance in human pathogens is clear, but the use of antimicrobials in animals may also be important. A number of reports have documented emerging resistance to multiple antimicrobial agents in verocytotoxin-producing *Escherichia coli* including *E. coli* O157:H7 (Kim, et al., 1994; Schroeder, et al., 2002). Since antimicrobials are not usually recommended for the management of *E. coli* O157:H7 infections in humans (Wong, et al., 2000), it is thought that the administration of sub-therapeutic levels of antimicrobials to animals may be the contributing factor and not just their use in humans that selects for this resistance. Resistant strains of *E. coli* O157:H7 were found in animal drinking water suggesting that water may act as reservoirs of infection for animals. Increasingly more data illustrates the emergence of multi-resistant food borne enteric pathogens transmitted to humans from farmed animals through the food chain (Levy, FitzGerald & Macone, 1976; Molback et al., 1999). These studies suggest that transmission of antimicrobial resistance from animals to humans occurs; however, they do not provide either overall estimates of how often this occurs, nor do they assist in understanding the mechanisms of transfer.

Humans may also be affected either directly through ingestion of water contaminated with antimicrobial resistant bacteria, or indirectly, through exposure to an environment or food that has been contaminated by the water (Leclerc, Schwartzbrod, & Dei-Cas, 2002; Lee et al., 2002). It is estimated that 25% to 75% of antimicrobials administered to animals can be excreted unaltered in faeces (Chee-Sanford et al, 2001). The use of animal faecal waste as fertilizer can result in ground water contamination. Goss, Barry, and Rudolph (1998) report that private wells on farms where manure is routinely applied have a greater occurrence of bacterial contamination than wells on farms without livestock. Runoff contaminated with faeces from sites of intensive animal rearing can percolate through soil close to wells. Additionally, waste from septic tanks and manure storage sites can seep or spill into surrounding watersheds or other ground water sources (McEwen & Fedorka-Cray, 2002).

In Ontario, groundwater provides about 30% of water requirements. Farm and rural families depend almost entirely on private wells. There are an estimated 500,000 private wells in Ontario (Goss, Barry, & Rudolph, 1998). Studies have found about 10% to 34% of private wells in Canada do not meet minimal standards for bacterial contamination (Johnston, 1985). Water contaminated with low *E. coli* counts (10 colony forming units /mL) has been associated with increased attack rates of gastrointestinal illness (Cabelli, Dufour, McCabe, & Levin, 1982). *Escherichia coli* gastroenteritis caused by drinking contaminated well water has been documented, including the recent outbreak in Walkerton, Ontario. (Hrudey et al, 2003; CCDR, 2000) Despite these risks, few studies have examined the role of antimicrobial resistant *E. coli* in drinking water.

In 1987-88, Kaspar et al. (1990) found that 32% and 9% of the *E. coli* collected from urban and rural surface waters respectively were resistant to one or more antimicrobial agents. McKeon et al. (1995) reported that 46% of *E. coli* found in rural, untreated water supplies were resistant to antimicrobials, with 14% being resistant to two or more agents. No speculations were made as to the origin of these strains.

More recently, Walia, Kaiser, Parkash, and Chaudhry (2004) found that 70%, 55% and 15% of *E. coli* (n=20) tested from drinking water in Michigan were resistant to carbenicillin, tetracycline, and streptomycin, respectively. Although the findings are based on a small sample size, the researchers reported that the antimicrobial resistant strains of *E. coli* from drinking water supplies were able to transmit resistance to non-resistant *E. coli* strains for ampicillin, tetracycline and streptomycin in conjugation studies. They also tested human urinary tract *E. coli* isolates from women living in the same region. Of the 20 human isolates tested, 42%, 44%, and 92% were resistant to carbenicillin, tetracycline, and streptomycin, respectively. Although they found the resistance rates for these antimicrobial agents to be similar between water and human *E. coli* for some antimicrobial agents, they did not establish whether the human acquisition of antimicrobial resistant *E. coli* was the result of consuming contaminated water.

We have recently initiated a two-year Canadian Institute of Health Research (CHIR)-funded (2004-2006) study entitled, *Prospective Multi-Province Surveillance for Antimicrobial-Resistant* Escherichia coli *in Drinking and Recreational Source Waters: Alberta, Ontario and Quebec*. This research will determine the baseline geographical prevalence of antimicrobial resistant *E. coli* in private drinking water and recreational/beach water sources in these provinces. Using geospatial mapping analyses, we will determine whether certain patterns of antibiotic resistance are clustered geographically to areas with high food animal/agriculture intensity and/or high human population densities. The information from this study will provide essential and much needed information about the prevalence of antibiotic resistant bacteria in Canadian waters and their potential impact on human health. We found that of 500 *E. coli* positive well samples collected over a just a three-month period in 2003 from the London and Hamilton regions, 12% of the *E. coli* were resistant to at least one of the antimicrobials tested, and 8% were resistant to two or more classes of antimicrobial agents. Preliminary 2004 data from the CIHR-funded study shows that of 410 *E. coli* positive well samples screened, 18% of the wells harboured *E. coli* isolates resistant to one antimicrobial agent, with 10% resistant to two or more. Of the resistant *E. coli* isolates, 43% were resistant to tetracycline, 25% to sulphamethoxazole, and 11% to streptomycin. These preliminary data emphasize the potential magnitude of the problem of antimicrobial resistant *E. coli* in private drinking water sources and further supports the role of water as a potential reservoir of antimicrobial resistant bacteria to humans. *We see a research opportunity to build upon the laboratory surveillance set up by the CIHR-funded study to address the potential human health threat posed by exposure to antimicrobial resistant E. coli in private drinking water sources.* To our knowledge, there have been no systematic and comprehensive studies to determine the human health impact of antimicrobial resistant bacteria in Canada in these water sources.

Antimicrobial resistance in human *E. coli* isolates is a rapidly emerging problem (McDonald et al., 2001). *Escherichia coli* are the ideal species for studying antimicrobial resistance in water. This organism does not occur naturally in the environment and is commonly used as a bacterial indicator for microbiologic water quality (McLellan, Daniels, & Salmore, 2001). *Escherichia coli* rank among the most predominant bacteria found in the human gastrointestinal tract and most *E. coli* is acquired through consumption of food or water. Gastrointestinal colonization with a wide variety of *E. coli* strains takes place soon after birth. Resistance has been found in both

nonpathogenic and pathogenic strains. The treatment of pathogenic strains (intestinal and extraintestinal) of *E. coli* is becoming more challenging as antimicrobial resistance becomes more common. Although most *E. coli* strains found in the gastrointestinal tract do not cause morbidity in otherwise healthy individuals, the fact that these strains have acquired resistance to a variety of antimicrobials is worrisome. Antimicrobial resistant *E. coli* have shown the ability to transfer resistance to other strains of *E. coli* as well as other organisms within the gastrointestinal tract (Bettelheim, 1997) and to acquire resistance from other organisms (Österblad et al., 2000; Oppegaard, Steinum, & Wasteson, 2001).

In the Toronto, Durham, and York regions of Ontario, several related outbreaks of infection (urinary tract, pneumonia, and bacteraemia) due to a single clone of multi-drug resistant *E. coli* were reported (Muller et al., 2002). Eight deaths were related to infection with the outbreak strain. Even with intensive infection control and careful management of antimicrobials, the strain continued to spread within the facilities. The plasmid associated with resistance in this clone has also been transmitted, in the gastrointestinal tract of colonized patients, to 12 other clones of *E. coli,* as well as strains of *Enterobacter cloacae, Citrobacter spp.*, and *Klebsiella pneumoniae.* These findings highlight the transferability of a resistance determinant to multiple bacterial species.

Pathogenic *E. coli* strains can contribute to gastrointestinal infections and extraintestinal infections such as abdominal, pelvic, blood-stream, and urinary tract infections (Russo & Johnson, 2003). It is estimated that *E. coli* causes 85 to 95% of all uncomplicated episodes of cystitis and pyelonephritis in women, with these bacteria originating from endogenous microflora of the gastrointestinal tract. Increasing resistance to sulfamethoxazole and ampicillin is being described. Manges et al. (2002) determined that 22% of the *E. coli* isolates from the urine of community-based subjects with cystitis were resistant to trimethoprim-sulfamethoxazole. These researchers found a single clone was responsible for over 30% of resistant isolates in three geographically disparate areas in the United States. They raised the potential for a common foodborne or waterborne source for the widespread dissemination of this resistant *E. coli* clone. Similarly, Burman et al. (2003) reported that 23% of urinary *E. coli* isolates were resistant to trimethoprim-sulfamethoxazole and concluded that it should not be considered the first drug of choice for uncomplicated urinary tract infections.

In Toronto, in urine cultures submitted from family medicine patients at the ten hospitals and clinics served by the Mount Sinai Hospital and Toronto Medical Laboratories in 2004, 34% of *E. coli* isolates were resistant to ampicillin and 21% were resistant to trimethoprim-sulfamethaxazole compared to 25% and 15%, respectively in 2002 (A. McGeer, personal communication, November 10, 2004). Amoxicillin and trimethoprim-sulfamethoxazole are the recommended first line agents for such infections (Ontario Anti-Infective Review Panel, 1994). Expert opinion suggests that when resistance to both agents is greater than 20%, first line therapy in adults should be changed to a fluoroquinolone. Thus, in Ontario, we need to switch to first-line fluoroquinolone therapy for community-based patients with cystitis, a switch that will likely be associated with rapid selection for resistance to fluoroquinolones, beyond which we have no effective therapy. These findings highlight the urgent need to understand sources and mechanisms of development of antimicrobial resistance.

In the London, Ontario region, antimicrobial susceptibility data for 2002-2003 show that for over 2,000 *E. coli* clinical isolates, 34% of city-wide hospital isolates were resistant to ampicillin, 30% to piperacillin, 18% to trimethoprim-sulfamethoxazole, 9% to gentamicin, and 6% to cefazolin (LLSG, 2003). These findings highlight the existence of antimicrobial resistant strains of *E. coli* in the proposed study region and support the need to study possible reservoirs

for antimicrobial resistant *E. coli*. They also correspond with the preliminary CIHR data that identified that 25% of *E. coli* isolates from water samples from the same region are resistant to sulfamethoxazole.

Strains of antimicrobial resistant *E. coli* have also been detected in a high proportion of faecal samples from healthy individuals in developed countries of the world. Resistance varies by country, sample, year, and type of antimicrobial agent. However, resistant strains of *E. coli* are common in both community-dwelling and hospital-dwelling subjects (Appendix A). Bruinsma, Hutchinson, et al. (2003) randomly sampled community-dwelling subjects in Canada, the Netherlands, and Greece. Faecal *E. coli* samples from 154 Canadian subjects showed lower rates of antimicrobial resistance than that in the other sites with 22% of isolates resistant to amoxicillin, 16% to oxytetracycline, 10% to trimethoprim, and 3% to cefazolin.

Studies following subjects over several months have reported that antimicrobial resistance patterns for faecal *E. coli* remained stable over time. Bruinsma, Filius et al. (2003) tested hospital patients' faecal samples at admission, discharge, and one and six months after discharge. Although there was an increase in the proportion of subjects with resistance to several antimicrobial agents between admission and six months after discharge, the differences were not statistically significant. Stürmer et al. (2004) followed community dwelling subjects for three months and noted that the prevalence of resistance remained stable for all antimicrobial agents tested. Of note, however, is that 13% of subjects who had a resistant strain of *E. coli* at baseline, had no resistant strain detected at three-month follow-up, while 12% of subjects had the opposite experience. No studies have looked at the factors related to changes in carriage of antimicrobial resistant strains of *E. coli*.

The correlation between antimicrobial resistance in colonizing and pathogenic strains makes the study of faecal colonization important in the understanding of the patterns of resistance, predicting future infecting isolates, and supporting antimicrobial choices in human and veterinary medicine. The study of antimicrobial resistant strains of *E. coli* in the faecal samples of healthy community subjects gives researchers a benchmark measure of the types of resistance present in the community.

Although the use of antimicrobials contributes to the selection of antimicrobial resistant *E. coli* in the human gastrointestinal tract, the question of whether the ingestion of antimicrobial resistant *E. coli* contaminated food or water might be a risk has never been addressed. We have found that drinking water from private wells can be contaminated with antimicrobial resistant *E. coli* making it a possible source of human acquisition of antimicrobial resistance that has, as yet, been unrecognized. Given that surveillance studies have shown that *E. coli* is the most common Gram-negative human pathogen and ranks among the most common nosocomial pathogens (Gordon, Jones, & SENTRY Participant Groups), and that antimicrobial resistance in *E. coli* is an emerging challenge, acquisition of antimicrobial resistant *E. coli* from water is an important human health problem that needs to be studied. Is there a correlation between antimicrobial resistant *E. coli* in drinking water and in human gastrointestinal tract following consumption of the contaminated water source?

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RESEARCH DESIGN

Systematic laboratory surveillance of over 6,000 samples of water from private wells in Southern Ontario for antimicrobial resistant *Escherichia coli* will be conducted over two years in a Canadian Institute of Health Research (CIHR)-funded study (PI, M. Louie, May 2004- May 2006). We would use this surveillance to identify contaminated wells and respective well owners. In the second year of the CIHR-funded study, to start May 2005, a nested case-control study design will be used to determine risk factors for well water contamination with antimicrobial resistant *E. coli*. We will also conduct a faecal carriage study to determine the impact of consumption of antimicrobial resistant *E. coli* contaminated well water on human faecal carriage and we will assess the persistence of faecal carriage of antimicrobial resistant *E. coli* strain(s) at baseline and at three months.

Susceptibility Testing

Laboratory Surveillance (Further details in Appendix B)

In brief, *E. coli* isolates from contaminated well water samples will be screened for antimicrobial resistance using agar plate method. Agar screen plates using cation supplemented Mueller Hinton media will each contain the following**:** amikacin 16μg/mL; gentamicin 4μg/mL; cephalothin 16 μg/mL; nalidixic acid 4 μg/mL; sulfamethoxazole 128 μg/mL; tetracycline 4 μg/mL; and streptomycin 32 μg/mL. Isolates growing on agar screen plates will be confirmed as *E. coli* isolates using standard biochemical tests and API-20E assay (Biomerieux Canada Inc.). Resistant isolates confirmed to be *E. coli* will be tested for their minimum inhibitory concentrations to antibiotics using the National Antimicrobial Resistance Monitoring System (NARMS) antimicrobial microbroth susceptibility panel at the Laboratory for Foodborne Zoonoses[1]. Since the NARMS panel does not adequately screen for extended-spectrum beta-lactamase (ESBL) resistance, ampicillin-resistant isolates will also be screened for the presence of ESBL resistance using cefpodoxime (4 μg/mL) agar screen plates and confirmed as ESBL producers using NCCLS methodology at the end of the study. Antimicrobial resistant *E. coli* will be defined as *E. coli* that is resistant to at least one antibiotic. All antimicrobial resistant *E. coli* isolates will be archived.

In year one of CIHR study, we found that the agar screen plate data correlated with the susceptibility data generated by the NARMS panel. To ensure that the turnaround will allow timely identification of eligible wells as to minimize recall bias during the telephone survey of well owner(s), we will use the agar screen plate data to identify wells contaminated with antimicrobial resistant *E. coli.* We will exclude those *E. coli* isolates if subsequent NARMS testing does not confirm the presence of resistance.

[1] NARMS –the National Antimicrobial Resistance Monitoring System for enteric bacteria in 1996 as a collaboration between CDC and the US Food and Drug Administration Center for Veterinary Medicine, and other US agriculture departments to prospectively monitor antimicrobial resistance in isolates of selected enteric bacteria from humans, animals, and animal products. The Canadian Integrated Program for Antimicrobial Surveillance (CIPARS) in collaboration with Health Canada, Canadian Food Inspection Agency and other partners has also adopted the NARMS susceptibility testing panel for surveillance of antimicrobial resistance in enteric bacteria of animal, food and human origin in Canada.

Faecal Carriage

Human rectal swab samples will be collected in Carey Blair transport media and sent to the study laboratory. Swabs will be re-suspended in TSB broth and incubated overnight. A one mL aliquot will be frozen at -70°C. Susceptibility testing will be performed after all swabs are collected and the laboratory will be blinded to the status (case well versus control well residence) of the participant. A swab of the frozen specimen will be screened for the presence of antimicrobial resistant *E. coli* using the agar screen plate susceptibility method described above, using MacConkey media with crystal violet. Up to five different morphotypes of lactose-fermenting colonies will be selected from the agar screen plates for further workup. We have validated this process to ensure that freezing would not affect recovery of antimicrobial resistant *E. coli* (M. Louie, personal communication, November 12, 2004).

Antimicrobial profiles from antimicrobial resistant *E. coli* from humans and matching private well water samples will be compared and tested to determine the relatedness of strains and resistance determinants (Appendix B).

Case Control Study of Wells

Inclusion/Exclusion Criteria

Inclusion criteria for the nested case-control study include: "wells" for which there is at least one adult, 18 years or older, who speaks English, and who has been a resident of the property at least two months. Exclusion criteria would include: if the well is already enrolled in the study or if no consent is obtained.

Definitions

The "case" under investigation will be defined as a private "well" contaminated with antimicrobial resistant *E. coli* (resistance to at least one class of antimicrobial agents) as detected using the methods described above. Two sets of controls will be used: "control" wells will be defined as 1) contaminated with non-resistant *E. coli* and 2) not contaminated with *E. coli.* Both control groups will be matched to case wells by week of water sample collection and county. "Submitter" refers to the person submitting the water sample for bacteriological testing. "Household member" refers to a person who lives on the property where the well is located.

Approach

Bacteriological testing of private well water sources is voluntary. To inform people submitting water samples for testing of the study, an information sheet (Appendix C) will be attached to all water sampling kits (bottle, data sheet, and tags) starting in March 2005. The information sheet will also be mailed along will all bacteriological test results by the testing laboratory during the study period. All private wells that have had a suitable sample submitted for bacteriological testing at the London and Hamilton regional public health laboratories between April 1, 2005 and March 31, 2006 will be eligible for the case-control section of the study.

The study coordinator will identify the case wells from weekly review of agar screen plate testing. S/he will then contact the Safe Drinking Water Unit at the Ministry of Health and Long-Term Care who is responsible for phoning people submitting samples that test positive for contamination. A staff member at the Safe Water Unit will call the people submitting samples from the selected wells to determine eligibility and to obtain permission for the study to contact

them. For logistic reasons, controls will be identified only after the case well has been enrolled in the study. We will identify three potential wells for each of the two control groups from which we can contact people to enroll in the study.

For both case and control wells, each well will be eligible to be enrolled only once into the study. Once permission to make contact is obtained, the study coordinator will contact the household of the person submitting the water sample from both case and control wells. Verbal consent for a face-to-face interview will be obtained and a site visit by a trained interviewer (same interviewer for all study sites) will be set up with the submitter of the water test or a designate of the submitter. Verbal consent will be obtained from the submitter to allow the study coordinator to contact his/her household members. Lag time to recruit and interview submitters of the case and control wells will be less than one month from the date of submission of the original water sample to minimize the impact of recall.

All interviews will conducted using a standardized "initial household" interview script (Appendix D) with the interviewer blinded to the status of case or control. Written consent forms will be required from all participants (Appendix E). For those who are not available for interview during the site visit, a telephone interview will be arranged, with verbal consent for the interview.

The interview will seek information from the submitter on residence type (e.g. home, farm, cottage), the well or other water source characteristics (type, age, depth, past test results, treatments), and age and type of septic system. If applicable, information on antibiotic use in farm animals and household pets will be asked.

During the site visit, the interviewer will take geospatial coordinates from the well head, estimate the distance between well and septic system, distance to the nearest farm (including tilled fields, fields with manure application, farm buildings, livestock pasture, housed animals, and species) and distance to other surface water (lake, stream, pond) (Appendix D).

Faecal Carriage Study

Inclusion/Exclusion Criteria

Inclusion criteria include: Submitters of case and control wells enrolled in the case-control study and their respective household members are eligible. Individuals must be 12 years of age or older, English speaking, full-time residents of the household for the last two months, and who consume the well water. For those under the age of 16, parental consent will be required along with the child's consent. Refusal to provide rectal swab does not exclude the individual from the questionnaire interview as long as other member(s) of the household have consented to submitting a rectal swab. Exclusion criteria: no consent.

Approach

At the end of the case-control (household) interview above, eligible persons (submitters and household members) representing case and control wells will be asked to participate in the study investigating links between antimicrobial resistance in well water and human colonization.

Eligible consenting household members will be asked further demographic and health related information using a standardized "initial personal" questionnaire (Appendix F) including: age, sex, occupation, co-morbid conditions (diabetes mellitus, neoplasia, etc.), medications, antimicrobial use in the last year, and hospitalization in the last year. Participants will be asked about gastrointestinal illness (vomiting or diarrhoea defined as loose stool or stool with abnormal liquidity, lasting one or more days) in the preceding three months. They will also be asked to recall the number, date, and type of antimicrobials taken in the past year. Other data (potential

covariates and confounding variables) will include recent travel, number of years at current residence, and tap water consumption. All participating subjects will be asked permission to contact them again in three months for a follow-up interview.

The second (3-month follow-up) household (Appendix G) and personal interviews (Appendix H), by a trained telephone interviewer, using standardized questionnaires will occur three months after the first (baseline) interview. We will determine current consumption of water from the private well, treatments made to the well water, as well as antimicrobial use, hospitalization, medications, gastrointestinal illness in the intervening three months. We will also determine any changes in the potential covariates and confounding variables, including presence of pets, recent travel, and changes in occupation.

Owners and household members from case wells and control wells will be asked to provide a rectal swab to test for antimicrobial resistant *E. coli*. Suitable sample containers and instructions for collection, storage, and submission of samples will be provided. Subjects will be asked to provide the sample at the time of the face-to-face interview to improve response rates. For the follow-up test, a second rectal swab collection kit will be mailed to participating submitter and participating household members to be returned to the study coordinator by post, three months after the first interview. The participant will be reminded at the time of the second interview. The second rectal swab will be used to determine if the subject has maintained, gained, or lost the antimicrobial resistant strain of *E. coli* within the three-month interval between samples.

Sample Size

In a recent study of rural groundwater quality, Conboy and Goss (2000) found that 80% of farms with wells at high risk of bacterial contamination housed livestock, while only 64% of farms with wells at low risk housed livestock. Assuming that approximately 20% of wells will be located on farms, and that approximately 10% of control premises will house livestock, a sample size of 300 pairs of matched cases with each set of controls would provide adequate power (80%) to detect increased risk of antimicrobial resistant *E. coli* contamination for water sources on premises housing livestock, compared with premises not housing livestock, with an Odds Ratio = 2.0.

For the faecal carriage study, we will recruit all willing household members from the 900 households enrolled in the case-control section of the study. We estimate that 70% (Akwar, Poppe, Pentney, & McEwen, 2000) of the estimated 1,890 adults (2.1 people 15 years and older per private household in study area: 2001 Census) in these households will consent to be interviewed and provide an initial rectal swab. We estimate that 70% of subjects completing the first interview will agree to participate in the follow-up study giving us a sample of about 1,300 for the faecal carriage study.

Analysis

All data from patient interviews will be entered in duplicate using Epi-Info 6.04d (CDC, Atlanta, Georgia, 2000). Descriptive statistics (frequency distributions, means, etc) of interview data and faecal data will be presented. We will also perform cluster analyses using the spatial scan statistic to assess spatially localized contamination. Spatial distributions of case and control wells will be analyzed using descriptive spatial statistics and spatial density measures. These analyses will be conducted using a geographic information system application (e.g. ArcGIS). Environmental risk factors (e.g. proximity to livestock operations) for private-well contamination with antimicrobial resistant *E. coli* will be identified initially using Fisher's Exact tests;

subsequently, conditional logistic regression will be used for multivariable analysis. The association between drinking water and human faecal antimicrobial resistant *E. coli* culture status will also be assessed using conditional logistic regression. A multivariable model with human faecal antimicrobial resistant *E. coli* culture status as the outcome and various health-related (e.g. prior antimicrobial use, occurrence of diarrhea) and demographic (e.g. age, occupation) covariates will be constructed, and drinking water antimicrobial resistant *E. coli* culture status will be offered into the model with appropriate adjustment for over-dispersion arising from multiple residents sampled per well. We will also investigate the use of an ordered outcome variable reflecting multiple resistance (e.g. polytomous or ordered logistic regression). Comparisons between the findings from the first and second faecal samples and interviews will be compared using dependent samples analyses. Turn-over tables from the susceptibility tests will be made to compare gross changes in susceptibilities from the first and second faecal swabs. Within and between groups analyses will also be conducted, as will log linear regression analyses with individual level effects modifications.

We will perform molecular characterization of antimicrobial resistant strains from faecal isolates over time compared to those from the respective well water samples.

ETHICS AND CONFIDENTIALITY

Ethics approval for research on human subjects is being submitted to the University of Western Ontario and University of Toronto research ethics boards. Ethics approval for the CIHR-laboratory surveillance components of the study are attached (Appendix I). Confidentiality of all patient and premise data will be strictly maintained. Databases will be kept password and firewall protected. Back up copies will be kept in locked filing cabinets in the office of the principal investigator. All data will be coded so that no databases will have patient identifiers. Interviewers will be blinded to the case/control status of the participants. The consent forms and logs linking coded data to patients will be kept in a separate locked filing cabinet.

Anticipated Value

This study will determine if proximity to livestock farms is an important risk factor for contamination of private drinking water sources with antimicrobial resistant *E. coli*. There remain high perceptions of regional contamination and risk exposure for enteric organisms such as *E. coli* in proximity to a variety of livestock settings. This will be the first comprehensive study to look at the impact of antimicrobial resistant *E. coli* from water sources on human health in Canada. The molecular analysis will establish any epidemiological links in those who consume contaminated well water. The faecal carriage study will give much-needed information on the longevity of carriage and thus, the increased potential for causing antibiotic-resistant extraintestinal infections. The proposed case/control and faecal carriage studies would not otherwise be possible without the support of the CIHR-funded laboratory surveillance component already in place.

TIMELINES / FEASIBILITY

December 2004	Submit Ethics for Case-Control and Faecal Carriage Studies in anticipation of funding
March 2005	Refine and pilot questionnaires, train interviewers Send out information sheets with water testing requisitions Inform local health regions of study
April 15/2005	Laboratory surveillance for private water starts
May 1/05 to Apr. 30/06	Contact with prospective subjects
May 15/05 to May 31/06	Initial interviews, collection of faecal swabs, environmental scan
Aug. 15/05 to Aug. 31/06	Second interviews and collection of follow-up faecal samples start
Nov 2005	Preliminary report to funding agency re: recruitment status
May to Dec 2006	Laboratory analysis of faecal samples
May 2006	Preliminary report to funding agency re: recruitment status
Jan to May 1/2007	Analysis of results Preparation of manuscripts Reports to funding agencies

LIMITATIONS

Submission of well water for bacteriological testing is voluntary in Ontario. Therefore, the samples sent for submission and therefore eligible for entry into the study may be sent from wells where the owner has a higher suspicion of contamination. We are, however, asking whether there has been gastrointestinal illness in the household in the previous three months and why they sent their water to be tested (routine, symptom-induced, or previous positive test) which will help determine if a higher suspicion of contamination was the reason for submission.

Although we are analysing both the well water and human faeces for the specific resistance patterns to determine their relationship, it is not possible to determine causality. It is possible that the water is not the carrier of the resistance to the human, but rather, that the human and the water source were both contaminated directly from the same point source (e.g. manure). We will, however, be able to reasonably eliminate the backward causation (human to septic system to water) through the site visit to determine location of well and septic system. The use of the control groups will also provide evidence to support or disprove the association between contaminated well water and human colonization.

The study is being conducted on drinking water from private wells for which the provincial government provides bacteriological testing without direct cost to the person submitting the sample. Although no treated urban control wells are being used, private wells with and without *E. coli* contamination are being used as controls. Since the study's purpose is to determine associations with antimicrobial resistance in drinking water and human carriage, we feel the controls are appropriate.

It is possible that wells that have a positive bacteriological water test will have been treated before the study personnel are able to visit the site to gather faecal samples. However, the researchers feel that the short period between possible treatment of the well water and collection of the swabs for laboratory testing will have little effect on the carriage of bacteria.

Due to the high costs for laboratory testing, it is only possible to test a portion of each water and faecal sample submitted. It is possible that other strains of *E. coli* exist within the sample but are not tested. Also due to the costs for testing, we are not requiring a water sample at the time of the second interview and rectal swab. However, owners of wells that are contaminated are asked, by the Ministry of Health and Long-Term Care, to submit subsequent samples from their wells to

determine its suitability for human consumption. We will be asking subjects to report the findings from well water samples sent for bacteriological testing between the first and second interviews during the second interview.

FUTURE DIRECTIONS

We will map and seek to characterize the spatial distribution of antimicrobial resistant *E. coli* in private wells within the area of study. Using the results of human faecal isolates analyses, we will attempt to spatially correlate the different resistant patterns of human source with the ones of drinking water source. We will perform spatial cluster analysis and will attempt to correlate the resistance patterns found in human isolates to land use and population attributes at various spatial aggregation levels.

Follow up of the participants in the faecal carriage study would provide valuable information regarding risk factors for colonization and factors associated with decolonization, if any. It would be possible, in a future study, to show that colonization with antimicrobial resistant *E. coli* is associated with higher rates of morbidity.

Another potential study would be perform spatial cluster analysis of antimicrobial resistance profiles from human faecal and water isolates from this study with data generated for *E. coli* resistance data from hospital and community acquired infections from patients living in the London and Hamilton regions.

APPENDIX A - RESISTANCE PATTERNS IN FECAL *Escherichia coli* ISOLATES

Study **Community dwelling**	Amox icillin	Trimeth oprim	Tetra cycline	Sulfameth axazole	Strepto mycin
Bonten, et al., 1992	16	29	20	44	26
London et al., 1994	10	9	21[1]	32	24
Österblad et al., 2000		9	14	16	18
Jonkers et al.,[α] 2002	28	22	27[1]		
Bruinsma et al.,[χ] 2003a	22	10	16[1]		
Bruinsma et al.,[α] 2003b	28	17	26[1]		
Stürmer et al., 2004					
Hospital patients					
Österblad et al., 2000		12	13	13	14
Jonkers et al.,[β] 2002	41	21	35[1]		
Bruinsma et al.,[β] 2003b	36	22	31[1]		

[1] Oxytetracycline
[2] Trimethoprim-Sulfamethaxazole
[3] Amoxicillin-clavulate
[α] Patients on admission
[β] Patients at discharge
[χ] Canadian subjects only

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APPENDIX B - SUSCEPTIBILITY TESTING AND MOLECULAR CHARACTERIZATION

Laboratory Surveillance – systematic and comprehensive laboratory surveillance for antimicrobial resistant *E. coli* in *E. coli* or coliform contaminated private well water.
Susceptibility Testing: Briefly, in Ontario, DC-agar plates are used for membrane filtration and a swab of all *E. coli* (blue colonies) will be archived. A single pool of colonies will enhance the likelihood of obtaining potential antimicrobial resistant isolates in an individual water sample. The swab will be placed in transport nutrient media (TSA slant), and shipped to the study laboratory. Screening for antibiotic resistance will be performed by the agar screen plate method (2). Selection of agar screen plates was based on pilot study on *E. coli* water isolates and antimicrobial resistance data from animal studies (3-5). Agar screen plates using cation supplemented Mueller Hinton media will each, contain the following: amikacin 16μg/mL; gentamicin 4μg/mL; cephalothin 16 μg/mL; nalidixic acid 4 μg/mL; sulfamethoxazole 128 μg/mL; tetracycline 4 μg/mL; and streptomycin 32 μg/mL. Isolates growing on agar screen plates will be confirmed as *E. coli* isolates using standard biochemical tests (citrate, indole, malonate metabolism) and API-20E (BioMerieux Canada Inc)(6). In the first year, *E. coli* isolates will be archived in skim milk and sent at monthly intervals to the Laboratory for Foodborne Zoonoses (LFZ, St. Hyacinthe) to be tested using the NARMS (National Antimicrobial Resistance Monitoring System, CDC) antimicrobial microbroth susceptibility panel (7). Isolates will be subcultured twice on blood agar plates prior to performing susceptibility testing. Since the NARMS panel does not adequately screen for extended-spectrum beta-lactamase (ESBL) resistance, ampicillin-resistant isolates will also be screened for the presence of ESBL resistance using cefpodoxime (4 μg/mL) agar screen plates and confirmed as ESBL producers using NCCLS methodology(8), (2). All AR-*E. coli* isolates will be archived. In the second year, for the private-well case-control study, agar screening will be done weekly, and *E. coli* strains will be sent to LFZ every two weeks for testing. This turnaround will allow timely identification of eligible wells as to minimize recall bias during the telephone survey of well-owner(s). Antimicrobial susceptibility results will be interpreted using resistance breakpoints relevant to human health as outlined by the National Committee for Clinical Laboratory Standards (9). AR-*E. coli* will be defined as *E. coli* that is resistant to at least one antibiotic.

Fecal Carriage Study: Rectal swabs will be collected from consenting participants. Swabs will be sent to the study laboratory in transport media. Upon receipt, the swabs will be inoculated into tryptic soy broth and incubated overnight. A one mL aliquot of the overnight broth will be archived and frozen at -70°C. Susceptibility testing will be performed at the end of the recruitment phase. A swab of the frozen sample will be inoculated onto MacConkey agar with crystal violet containing the following antibiotic concentrations (μg/mL): amikacin 16μg/mL; gentamicin 4μg/mL; cephalothin 16 μg/mL; nalidixic acid 4 μg/mL; sulfamethoxazole 128 μg/mL; tetracycline 4 μg/mL; and streptomycin 32 μg/mL. Lactose-fermenting colonies (up to 5 different morphotypes) growing on the antibiotic screen plates will be screened as presumptive *E. coli* isolates (citrate, indole, malonate metabolism). *Escherichia coli* confirmation will done using API-E20 (BioMerieux) assay. Antimicrobial resistant *E. coli* will be archived in skim milk and sent to the Laboratory for Foodborne Zoonoses (LFZ, St. Hyacinthe) to be tested using the standardized NARMS (National Antimicrobial Resistance Monitoring System, CDC) antimicrobial microbroth susceptibility panel to determine the minimum inhibitory

concentrations for the following antibiotics: amikacin, amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulphamethoxazole, tetracycline, trimethoprim/sulphamethoxazole. Detection for extended-spectrum beta-lactamase (ESBL) resistance, will be done as described above.

Molecular Characterization Studies.

Rationale: Molecular mechanisms of drug resistance are complex and multiple genetic mechanisms may exist for a particular resistance phenotype in the same species (10). Although resistance to antimicrobials in bacteria can be either intrinsic or acquired, it is the acquired resistance that is important in this study. Acquired resistance can arise due to genetic changes through spontaneous mutations involving genes existing in the organism or via genetic rearrangement of the normal complement of genes. The second mechanism involves the acquisition of resistance genes through horizontal gene transfer. It is beyond the scope of this study to identify all possible mechanisms of antimicrobial resistance in the *E. coli* strains isolated. Based on previous farm animal studies and the pilot study of water *E. coli* isolates, the most frequently reported antimicrobial agents against which *E. coli* are resistant include: tetracycline, sulfamethoxazole, streptomycin and ampicillin (3-5).

Molecular Methods: ***Plasmid Profiling and Transformation Studies:*** Plasmid profiling will be conducted on specific isolates to determine the clonality of resistance plasmids. This is particularly useful when characterizing the multidrug resistance phenotypes associated with many R-plasmids (11). To identify the plasmid that carries the resistance, plasmid DNA harbouring the putative resistance marker will be extracted (Qiagen) and then transferred by electroporation to a recipient strain (DH10B, Invitrogen) that does not contain any plasmid elements. Plasmid DNA will then be purified from the transformant and fingerprints will be generated using a specific restriction enzyme(s) (12). These fingerprints can then be compared to determine if a plasmid is spreading between strains and within animals and humans. Susceptibility testing of the transformant(s) will be done to determine the antibiogram(s) and to determine the number of resistance elements residing on the plasmid of interest.

PCR Detection: A PCR mutiplex assay will be used on selected and representative isolates to identify the tetracycline resistance genes commonly found in Gram-negative bacteria (13). Genes conferring the extended-spectrum beta-lactamase (ESBL) phenotype and Class 1 integrons in *Enterobacteriaceae* will be detected and characterized using a combination of PCR and sequence analysis (12), (14). Presence of the *sulI* gene that confers resistance to sulfamethoxazole and is often associated with class 1 integrons, will be detected using PCR as previously described (15). *Plasmid*-mediated Ambler Class C resistance will be detected using a multiplex assay (16). Up-regulation of chromosomal *ampC* may account for the AmpC resistance phenotype in *E. coli* and we will measure this using a reverse transcriptase real-time semi-quantitative PCR (Taq-Man) for the *E. coli* AmpC gene (Mulvey, unpublished data). Nalidixic acid and/or ciprofloxacin resistant *E. coli* strains will be analyzed for mutations in the *Gyr*A and *Par*C genes (17), (18). Aminoglycoside resistance will also be determined on selected isolates (18), (10). *Other resistance phenotypes identified during the course of the study will be subjected to further molecular characterization as appropriate given that we do not know the full spectrum of antimicrobial resistance in E. coli from water sources at this time.*

Molecular Characterization of Virulence Determinants: Although *E. coli* is a natural part of the human gut flora, specific strains can acquire genetic determinants that enable them to become pathogenic to both humans and animals. Pathogenic *E. coli* can cause either extraintestinal disease (urinary tract infections, or sepsis and meningitis) (19) or intestinal disease (diarrheal disease) (20). To date over a 100 virulence genes have been associated with pathogenic *E. coli*, making it extremely time consuming and costly to determine the pathotype of a particular strain using conventional methods such as PCR. Recently Bekal et al., have used microarray technology to develop a "pathochip" capable of detecting all 105 known virulence factors (1). Dr. C. Clark (National Microbiology Laboratory, NML, Health Canada) has received funding from Health Canada to develop an enhanced pathochip in collaboration with Bekal's group. We, in collaboration with Dr. C. Clark will determine the pathotype for the *E. coli* strains identified in this study. AR-*E. coli* isolates from the case-control study will be studied for potential virulence factors. The information gained in this portion of the study will be combined with the antimicrobial resistance data. By doing this, we will not only identify antimicrobial resistant strains, but also determine the frequency of resistant and virulent strains capable of causing disease that may require antimicrobial therapy.

Strain Subtyping: AR-*E. coli* strains from both the case-wells and the fecal samples of the matched well-owner/household member(s) will be subtyped using pulsed field gel electrophoresis (PFGE), a standardized method of typing, used by PulseNet USA and PulseNet Canada for typing enteric/foodborne pathogens (21-22). DNA extraction and PFGE analysis will be performed as previously described (23). Briefly, *Xba*I (Invitrogen, Burlington, ON) will be used to digest genomic DNA and PFGE will be done using a CHEF-DRIII apparatus (Bio-Rad Laboratories, Richmond, CA) @ 6V/cm and 14°C for 19 hours with switch times ranging from 2.2 to 54.2 seconds. The gel will be visualized following ethidium bromide staining under UV light. The image will be entered into BioNumerics V2.5 Software (Applied Maths, Austin, TX). Cluster analysis for relatedness will be assessed by the visual method of Tenover (24) and by computer-assisted application of the Dice coefficient to bands in the gel (130). For clustering, the un-weighted pair group method with arithmetic means will be used, with a band position tolerance of 1.0%. For this analysis, isolates will be considered to be identical if their DNA profiles are identical. Isolates will considered related if the Dice coefficient >90%. There are no criteria for analyzing PFGE profiles for *E. coli*; we have arbitrarily chosen a 90% cutoff value solely for this study.

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