

Aichi virus in Alberta, Canada: a one year examination of human diarrheic stool samples by RT-PCR

A. Houde¹, E. Poitras¹, D. Leblanc¹, P. Ward¹, G.D. Inglis², V.F. Boras³, F. Loisy-Hamon⁴ and B. Lebeau⁴

¹ Agriculture and Agri-Food Canada, Food Research and Development Centre, 3600 Casavant Blvd. West, St. Hyacinthe, Quebec, Canada J2S 8E3

² Agriculture and Agri-Food Canada, Lethbridge Research Centre 5403-1st Avenue S, Lethbridge, Alberta, Canada T1J 4B1

³ Department of Laboratory Medicine, Chinook Regional Hospital, 960-19th Street S, Lethbridge, Alberta, Canada T1J 1W5

⁴ CEERAM SAS, 1 allée de la Filée, B.P. 54424, 44244 La Chapelle sur Erdre, France

Aichi virus (AiV) was first recognized in 1989 as the likely cause of oyster-associated gastroenteritis in Japan. Since then, this virus has been associated with gastroenteritis in adults and children in different countries. Stool samples were collected from 960 acute diarrheic individuals (80 samples per month) from May 2008 to April 2009 in the Chinook Health Region in the province of Alberta, Canada. For each month, the distribution of diarrheic samples was: 20 samples from individuals between 0 and 16 years of age, 40 samples from individuals between 17 and 60 years of age, and 20 samples from individuals over 60 years of age. In addition, 28 stool samples from healthy individuals (i.e. no recent evidence of gastroenteritis) were tested as controls. AiV RNA was detected by two different reverse transcription-polymerase chain reaction (RT-PCR) systems: a conventional system, and a commercial real-time detection kit. No AiV RNA was found in the 988 samples tested with the conventional RT-PCR assay while one sample from a diarrheic individual tested positive for AiV RNA using the commercial real-time RT-PCR detection kit. This result was confirmed by sequencing. The low incidence (0.1%) of AiV in samples from diarrheic humans indicates that this virus is not a significant causative agent of gastroenteritis in Southwestern Alberta, Canada.

Keywords Aichi virus, detection, RT-PCR, real-time RT-PCR

1. Introduction

Viral gastroenteritis is a common illness that affects humans of all ages. Norovirus (NoV), rotavirus (RV), sapovirus (SaV), astrovirus (HAstV) and enteric adenovirus (types 40 and 41) are considered as the main viral agents associated with gastroenteritis and diarrhea [1]. However, in many gastroenteritis outbreaks, the causative agent remains unidentified and other viruses were suggested as possible aetiological agents. Aichi virus (AiV) has been proposed as a novel causative agent of acute gastroenteritis. This virus was first recognized in 1989 as the likely cause of oyster-associated gastroenteritis in Japanese patients [2]. Since then, this virus has been associated with gastroenteritis in adults and children in several countries in Asia [3-6], Europe [7-12], Northern Africa [13, 14] and South America [7].

AiV is classified in the family *Picornaviridae* which contains twelve currently recognized genera: *Aphthovirus*, *Avihepatovirus*, *Cardiovirus*, *Enterovirus* (which is including *Rhinovirus*), *Erbovirus*, *Hepatovirus*, *Kobuvirus*, *Parechovirus*, *Sapelovirus*, *Senecavirus*, *Teschovirus* and *Tremovirus* [15]. This small, round and non-enveloped virus of about 30 nm in diameter [2] belongs to the genus *Kobuvirus* that also includes a second species, *Bovine Kobuvirus* [16, 17]. Recently a third candidate species of *Kobuvirus* has been described in pigs [18-20]. The AiV genome consists of a single-stranded positive-sense RNA molecule of approximately 8.3 kb terminated by a poly(A) tail [21]. The viral RNA contains a single open reading frame translated into a large polyprotein of 2432 amino acids that is cleaved by encoded viral proteases into capsid (VP0, VP1 and VP3) and non structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D).

AiV was first subdivided into two different genotypes (A and B) based on a 519 bp sequence located at the 3C/3D junction [22]. Recently, a third genotype (C) was proposed. Phylogenetic analysis of 13 AiV strains using a 699 bp sequence of the VP1 gene showed a good correlation with the 3C/3D classification system and supported the proposed third genotype [9].

A high prevalence of antibodies against AiV was reported in Japanese (55%) [3], German (76%) [7] and Spanish (70%) [23] populations. The seroprevalence of antibodies against AiV increases with age but seems to reach a plateau at the age of 35 years with 83% of positive sera in Japan [3], at the age of 15 years with 86% of positive sera in Germany [7], at the age of 30 years with 84% of positive sera in France [24], and at the age of 20 years with 85% of positive sera in Spain [23]. It was suggested that these differences may be due to different patterns in the epidemiology of AiV within these countries.

Until now, most studies have used conventional reverse transcription-PCR (RT-PCR) for the detection of AiV's RNA. RT-PCR primers are designed at the 3C/3D junction of AiV genome and used in conventional RT-PCR (conRT-PCR) [22]. The primer pair 6261-6779 system produces a 519 bp cDNA fragment that is also used for

molecular genotyping of the AiV strains. The detection of AiV RNA is also performed using variant systems within this genomic region by including primer pairs 6309-6488 [7] or C94b-246k [5] in nested RT-PCR assays. Although conRT-PCR is currently the primary AiV detection method, post-amplification steps, such as gel electrophoresis combined to DNA probe hybridization or sequencing, are still necessary to confirm the amplification products and to prevent the misinterpretation of false positive results due to non-specific amplification. Real-time PCR technology offers the possibility of simultaneously amplifying and detecting the targeted nucleic acids in a single step procedure, thereby eliminating the need for post-amplification processing steps. The detection step can be performed by the integration of a specific linear dual-labelled oligoprobe in the amplification mastermix and is achieved during the exponential phase of the targeted sequence amplification. Therefore, this technology offers many advantages over conRT-PCR for rapid and specific detection of viral particles, including 1) high sensitivity; 2) as mentioned above, the possibility of simultaneously amplifying and detecting the targeted nucleic acids in a single step procedure; 3) a wide dynamic range ($> 10^7$ fold) allowing a straightforward comparison between samples containing a large range of viral RNA concentrations; 4) the possibility of generating quantitative data; and 5) reduced risk of carry-over contamination through the use of a closed system [25-28]. For these reasons, real-time RT-PCR is now largely used for the detection of enterically transmitted viruses in food, environmental and clinical samples (for a recent review [29]). For the detection of AiV, the only real-time RT-PCR system currently available is the proprietary detection kit "aichi@ceeramTools@.Health" commercialized by the "Centre Européen d'Expertise et de Recherche sur les Agents Microbiens (CEERAM)" [30].

No data on the presence and the prevalence of AiV are available in Canada. The Chinook Health Region (CHR) is located in Southwestern Alberta, Canada. This region possesses a high rate of enteritis amongst its human inhabitants [31]. Similarly to the Hungarian study [11], the CHR is landlocked and seafood, including oysters and shellfish, is not commonly consumed. However, this region possesses one of the highest densities of livestock in North America [32].

The aim of this study was to determine the incidence and the contribution of Aichi viruses to diarrheal diseases in the CHR community in the province of Alberta (Canada) over a one year period.

2. Materials and Methods

2.1 Stool samples

A total of 960 stool samples from human exhibiting signs of gastroenteritis and 28 samples from non-diarrheic individuals (healthy volunteers) as controls were collected over a twelve month period from April 2008 to March 2009. For each month, 80 samples from three age groups (A, B, and C) were randomly selected. Group A stools were from individuals ≤ 16 years of age with five samples (20 total) chosen from each of the following age categories: < 1 year; 1 to 5 years; 6 to 10 years; and 11 to 16 years. Group B stools were from individuals between 17 and 60 years of age with ten samples (40 total) chosen from each of the following age categories: 17 to 30 years; 31 to 40 years; 41 to 50 years; and 51 to 60 years. Group C stools were obtained from individuals > 60 years of age with samples (20 total) obtained from individuals in the following age categories: 61 to 70 years ($n=5$); 71 to 80 years ($n=10$); and >80 years ($n=5$).

2.2 Viral RNA isolation from faecal material

Faecal samples were diluted 1:5 (w/v) in sterile PBS, pH 7.2 (Invitrogen, Burlington, ON, CA) before centrifugation of 20 min at $4,000 \times g$. The stool suspensions were adjusted to reach 1% sodium dodecyl sulphate (Sigma-Aldrich, Oakville ON, Canada) and 100 $\mu\text{g}/\text{mL}$ of Proteinase K (Qiagen, Mississauga, ON, Canada). Mixtures were incubated at 37°C for 1 h. In order to monitor the recovery of viral particles and the RNA extraction process, 3.2×10^3 pfu of the feline calicivirus (FCV) were added to 140 μl of the resulting suspensions as sample process control (SPC). Viral RNA was extracted with QIAamp Viral RNA mini (Qiagen) protocols adapted for the QIAcube robotic workstation (Qiagen) using QIAamp Viral RNA body fluid: manual lysis protocol. In order to protect the extracted RNA from exogenous RNases, RNase inhibitor (RNaseOut, Invitrogen) was added to the final AVE elution buffer. RNA solutions were stored at -80°C until use.

2.3 TaqMan real-time RT-PCR assays for the detection of FCV and Aichi virus

The TaqMan assay for the detection of FCV was carried out in 25 μl of a reaction mixture with 2.5 μl of extracted RNA and 22.5 μl of master mix. Master mix was prepared using the 1-step Brilliant II QRT-PCR core reagent™ kit, (Stratagene, La Jolla, CA, USA) containing 5.0 mM of MgCl_2 , 300 nM of FCV forward and reverse primers, and 200 nM of TaqMan probe as described previously [33, 34]. Real-time RT-PCR amplifications were performed on a Stratagene Mx3005P (Stratagene) in a 96-well format under the following conditions: 30 min at 50°C for reverse transcription, 95°C for 10 min for reverse transcriptase inactivation and initial denaturation, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

Detection of AiV was also performed using the commercial detection kit aichi@ceeramTools®.Health (Ceeram S.A.S., La Chapelle sur Erdre, France) which includes its own internal amplification control according to the manufacturer's instructions [30].

2.4 Conventional RT-PCR

ConRT-PCR reactions were performed in a total volume of 25 µl using the Qiagen One-Step RT-PCR™ kit according to the manufacturer's recommendations in an Eppendorf Mastercycler gradient system (Brinkman Instruments Canada Ltd., Mississauga, ON, Canada). Extracted RNA (5 µl) was used as template. The conRT-PCR primers C94b and 246k at the 3C/3D junction of AiV genome were used at a final concentration of 500 nM each as previously described [22]. Reverse transcription was at 50°C for 30 min, followed by a 15 min denaturation step at 95°C. PCR amplification consisted of 45 cycles of amplification with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min followed by a final cycle of incubation at 72°C for 10 min. All the RT-PCR products were resolved in 1.5% (w/v) agarose gel (Invitrogen), and bands were visualized by ethidium bromide staining.

Since no AiV strains were available as positive control for the conRT-PCR assay, an artificial template corresponding to the sequence of the AiV reference strain A846/88 (GenBank # NC_001918) between nucleotides 6398 and 6663 was synthesized and included in pIDTSMART-KAN vector (IDT, Coralville, IA, USA). Purified plasmids were then used as positive controls for the conRT-PCR assay.

2.5 Cloning and sequencing

Amplicons were cloned into pCR 2.1 TOPO vector using TOPO TA Cloning kit (Invitrogen) with TOP10 electrocompetent cells in accordance with the manufacturer's recommendations. To confirm the identity of the amplicon, sequencing was performed on recombinant plasmids in both directions using a CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) and a CEQ Dye Terminator Cycle sequencing kit (Beckman Coulter) using M13 forward and reverse primers.

3. Results

As only 15 diarrheic stools were available from individuals under 16 years of age in the month of March 2009, five randomly selected diarrheic stool samples from individuals in the closest age group (between 17 and 30 years of age) were added in the study. For each set of 60 RNA extractions, two extractions with sterile PBS were included to confirm the sterility of the extraction solutions. Each molecular detection assay included a negative control (RNase free water) and a positive control (cloned artificial template for conRT-PCR, cloned amplicon for real-time RT-PCR detection of FCV, and the positive control included in the commercial kit for the detection of AiV). Negative controls were consistently negative in all assays. Positive controls (including the internal control in the AiV commercial detection kit) were detected in all real-time RT-PCR and conRT-PCR assays. All extracted RNA samples were first individually tested for FCV. FCV RNA was detected in all samples showing the efficiency of the sample extraction process and that RT-PCR reactions were not affected by inhibitors (Table 1).

A total of 960 stool samples from human exhibiting signs of gastroenteritis and 28 samples from non-diarrheic individuals collected over a 12 month period were screened for the presence of AiV. No AiV RNA was detected with the conRT-PCR assay. However, one sample was positive for AiV RNA using the commercial real-time RT-PCR detection kit aichi@ceeramTools®.Health (Table 1) representing a 0.1% incidence of infection amongst diarrheic individuals in the CHR. The one AiV positive sample was obtained from a 35-year-old woman in November 2008. Despite many attempts, the conRT-PCR assay failed in producing an amplicon. Therefore, the amplicon produced using the commercial kit was confirmed as AiV (isolate CAN-01) by the nucleotide sequence and was determined to possess a 95 to 98% nucleotide-sequence similarity with 91 AiV isolates available in GenBank (data not shown).

Table 1 Detection of Aichi virus by conventional RT-PCR and commercial real-time RT-PCR in 988 human stools from Southwestern Alberta, Canada.

	Group A ¹ N = 235	Group B ² N = 485	Group C ³ N = 240	Healthy ⁴ N = 28
Sample Process Control (FCV)	235	485	240	28
Real-time RT-PCR				
Aichi virus conRT-PCR	0	0	0	0
Aichi virus Commercial real-time RT-PCR kit ⁵	0	1	0	0

¹ ≤ 16 years of age

² 17 to 60 years of age

³ > 60 years of age

⁴ non-diarrheic volunteers

⁵ commercial detection kit aichi@ceeramTools®.Health (Ceeram)

4. Discussion

No AiV RNA was detected in the 28 stool samples obtained from the healthy individuals by both RT-PCR assays. The conRT-PCR amplification system, based on primers C94b and 246k located at the 3C/3D genomic region did not detect AiV RNA in the 960 stool samples examined from diarrheic individuals. However, AiV RNA was detected in one sample at a threshold cycle value (C_t) of 37.5 using the commercial real-time RT-PCR detection kit aichi@ceeramTools®.Health in the one diarrheic stool. The identity of AiV was confirmed and the amplicon shared a 95 to 98% nucleotide-sequence similarity with 91 AiV isolates available in GenBank, including the reference strain A846/88 (97%). Since a reliable phylogenetic tree could not be constructed due to the small size of the nucleotide sequence obtained (TaqMan amplicon of approximately 90 bp), it was not possible to assign a formal genotype to the Canadian AiV isolate, CAN-01.

The high C_t value observed for the AiV positive sample suggests that the virus was present at a low concentration (i.e. between 1 and 10 genomic copies). Although 5 µl of extracted RNA was used for RT-PCR (i.e. compared to 2.5 µl used for the real-time assay), it is possible that the conRT-PCR system failed to detect AiV RNA in the sample because of the lower sensitivity of RT-PCR relative to real-time RT-PCR assays. Another possible explanation for the inability of conRT-PCR to detect the AiV may be the result of mismatches in the primer annealing regions of the Canadian AiV RNA. This is currently under evaluation.

The AiV positive stool sample was collected from a 35-year-old woman in November 2008. This diarrheic sample was further tested for other viral agents associated with gastroenteritis. No NoV GI, NoV GII, NoV GIV, SaV, RV or astrovirus RNA was detected within this sample by real-time RT-PCR (data not shown). However, co-infection with a bacterial pathogen cannot be excluded, and this is currently being determined. In some countries, seroprevalence studies have shown that up to 80 to 95% of individuals of 30 to 40 years of age produce antibodies against AiV [3, 7, 23, 24]. Although AiV is detected in sporadic cases of gastroenteritis in adults, it is believed that exposure to AiV mainly occurs during childhood [6, 7]. The low incidence (0.1%; 1/960) of AiV that we observed in diarrheic stools in the current study is consistent with the low incidence of infection reported in other studies. For example, AiV was detected in 2.3% (5/222) of children in Pakistan [4], 0.9% of hospitalized children in France [9], 1.5% (1/65) of children in Hungary [11], 0.5% (5/1063) of children in Finland [10], 3.5% (22/632) of children in Tunisia [13], 1.8% (8/445) of children in China [6], and 3.1% (28/912) of adults and children with diarrhea in Japan, Bangladesh, Thailand and Vietnam [5]. No AiV was detected in 941 stools obtained from individuals with gastroenteritis during outbreaks over a 10-year period in the Netherlands [1].

The high seroprevalence of AiV antibodies in the population compared to the low detection rate in cases of gastroenteritis raises questions about the aetiology, virulence, and pathogenicity of AiV in symptomatic gastroenteritis [7, 9, 11]. AiV was also identified in the faecal sample of a 3-year-old child suffering from respiratory disease (i.e. pharyngitis, obstructive bronchitis, rhinitis and, finally, bronchopneumonia) and purulent conjunctivitis in addition to diarrhea [11]. AiV antigen has also been detected in a non-diarrheic stool from a child suffering from respiratory disease in Japan [3]. Reuter et al. [11] proposed two possible hypotheses: 1) AiV infection may usually be asymptomatic; and 2) AiV infection is symptomatic, but is also associated with other symptoms rather than gastroenteritis. Further studies must be conducted to determine the clinical significance of these viruses.

In conclusion, this study reports the first detection of AiV in Canada. The AiV RNA was detected using a commercial real-time RT-PCR detection kit in a diarrheic stool sample obtained from a 35-year-old woman exhibiting

signs of gastroenteritis in November 2008. The low incidence (0.1%) of AiV in the 960 samples obtained from diarrheic humans indicates that this virus is not a significant causative agent of gastroenteritis in Southwestern Alberta, Canada.

Acknowledgements We wish to thank the staff of the Chinook Regional Hospital Department of Laboratory Medicine and Kathaleen House (Agriculture and Agri-Food Canada, Lethbridge Research Centre) for processing stool samples. We also wish to thank all of the non-diarrheic volunteers for providing stool samples and for their interest in the study. The support by Agriculture and Agri-Food Canada's Risk Mitigation Strategy Program (RBPI 233) is gratefully acknowledged.

References

- [1] Svraka S, Duizer E, Vennema H, de Bruin E, van der Veer B, Dorresteyn B, Koopmans M. Etiological role of viruses in outbreaks of acute gastroenteritis in the Netherlands from 1994 through 2005. *Journal of Clinical Microbiology*. 2007;45:1389-1394.
- [2] Yamashita T, Kobayashi S, Sakae K, Nakata S, Chiba S, Ishihara Y, Isomura S. Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis. *Journal of Infectious Diseases*. 1991;164:954-957.
- [3] Yamashita T, Sakae K, Ishihara Y, Isomura S, Utagawa E. Prevalence of newly isolated, cytopathic small round virus (Aichi strain) in Japan. *Journal of Clinical Microbiology*. 1993;31:2938-2943.
- [4] Yamashita T, Sakae K, Kobayashi S, Ishihara Y, Miyake T, Mubina A, Isomura S. Isolation of cytopathic small round virus (Aichi virus) from Pakistani children and Japanese travelers from southeast Asia. *Microbiology and Immunology*. 1995;39:433-435.
- [5] Pham NT, Khamrin P, Nguyen TA, Kanti DS, Phan TG, Okitsu S, Ushijima H. Isolation and molecular characterization of Aichi viruses from fecal specimens collected in Japan, Bangladesh, Thailand, and Vietnam. *Journal of Clinical Microbiology*. 2007;45:2287-2288.
- [6] Yang S, Zhang W, Shen Q, Yang Z, Zhu J, Cui L, Hua X. Aichi virus strains in children with gastroenteritis, China. *Emerging Infectious Diseases*. 2009;15:1703-1705.
- [7] Oh DY, Silva PA, Hauroeder B, Diedrich S, Cardoso DD, Schreier E. Molecular characterization of the first Aichi viruses isolated in Europe and South America. *Archives of Virology*. 2006;151:1199-1206.
- [8] Le Guyader FS, Le Saux JC, Ambert-Balay K, Krol J, Serais S, Parnaudeau S, Giraudon H, Delmas G, Pommepey M, Pothier P, Atmar RL. Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak. *Journal of Clinical Microbiology*. 2008;46:4011-4017.
- [9] Ambert-Balay K, Lorrot M, Bon F, Giraudon H, Kaplon J, Wolfér M, Lebon P, Gendrel D, Pothier P. Prevalence and genetic diversity of Aichi virus strains in stool samples from community and hospitalized patients. *Journal of Clinical Microbiology*. 2008;46:1252-1258.
- [10] Kaikkonen S, Rasanen S, Ramet M, Vesikari T. Aichi virus infection in children with acute gastroenteritis in Finland. *Epidemiology and Infection*. 2009; doi:10.1017/S0950268809991300.
- [11] Reuter G, Boldizar A, Papp G, Pankovics P. Detection of Aichi virus shedding in a child with enteric and extraintestinal symptoms in Hungary. *Archives of Virology*. 2009;154:1529-1532.
- [12] Rasanen S, Lappalainen S, Kaikkonen S, Hamalainen M, Salminen M, Vesikari T. Mixed viral infections causing acute gastroenteritis in children in a waterborne outbreak. *Epidemiology and Infection*. 2010; doi:10.1017/S0950268809991671.
- [13] Sdiri-Loulizi K, Gharbi-Khelifi H, de Rougemont A, Chouchane S, Sakly N, Ambert-Balay K, Hassine M, Guediche MN, Aouni M, Pothier P. Acute infantile gastroenteritis associated with human enteric viruses in Tunisia. *Journal of Clinical Microbiology*. 2008;46:1349-1355.
- [14] Sdiri-Loulizi K, Hassine M, Gharbi-Khelifi H, Sakly N, Chouchane S, Guediche MN, Pothier P, Aouni M, Ambert-Balay K. Detection and genomic characterization of Aichi viruses in stool samples from children in Monastir, Tunisia. *Journal of Clinical Microbiology*. 2009;47:2275-2278.
- [15] International Committee on Taxonomy of Viruses (ICTV). Virus Taxonomy: 2009 Release. Available at: <http://ictvonline.org/virusTaxonomy.asp?version=2009>. Accessed May 6, 2010.
- [16] Yamashita T, Ito M, Kabashima Y, Tsuzuki H, Fujiura A, Sakae K. Isolation and characterization of a new species of kobuvirus associated with cattle. *Journal of General Virology*. 2003;84:3069-3077.
- [17] Khamrin P, Maneekarn N, Peerakome S, Okitsu S, Mizuguchi M, Ushijima H. Bovine kobuvirus from cattle with diarrhea. *Emerging Infectious Diseases*. 2008;14:985-986.
- [18] Reuter G, Boldizar A, Kiss I, Pankovics P. Candidate new species of *Kobuvirus* in porcine hosts. *Emerging Infectious Diseases*. 2008;14:1968-1970.
- [19] Yu JM, Jin M, Zhang Q, Li HY, Li DD, Zu ZQ, Li JS, Cui SX, Yang SH, Liu N, Duan ZJ. Candidate porcine *Kobuvirus*, China. *Emerging Infectious Diseases*. 2009;15:823-825.
- [20] Khamrin P, Maneekarn N, Kongkaew A, Okitsu S, Ushijima H. Porcine kobuvirus in piglets, Thailand. *Emerging Infectious Diseases*. 2009;15:2075-2076.
- [21] Yamashita T, Sakae K, Tsuzuki H, Suzuki Y, Ishikawa N, Takeda N, Miyamura T, Yamazaki S. Complete nucleotide sequence and genetic organization of Aichi virus, a distinct member of the *Picornaviridae* associated with acute gastroenteritis in humans. *Journal of Virology*. 1998;72:8408-8412.
- [22] Yamashita T, Sugiyama M, Tsuzuki H, Sakae K, Suzuki Y, Miyazaki V. Application of a reverse transcription-PCR for identification and differentiation of Aichi virus, a new member of the Picornavirus family associated with gastroenteritis in humans. *Journal of Clinical Microbiology*. 2000;38:2955-2961.
- [23] Ribes JM, Montava R, Tellez-Castillo CJ, Fernandez-Jimenez M, Buesa J. Seroprevalence of Aichi virus in a Spanish population from 2007 to 2008. *Clinical and Vaccine Immunology*. 2010;17:545-549.

- [24] Goyer M, Aho LS, Bour JB, Ambert-Balay K, Pothier P. Seroprevalence distribution of Aichi virus among a French population in 2006-2007. *Archives of Virology*. 2008;153:1171-1174.
- [25] Mackay IM, Arden KE, Nitsche A. Real time PCR in virology. *Nucleic Acids Research*. 2002;30:1292-1305.
- [26] Poitras E, Houde A. La PCR en temps réel: principes et applications. *Reviews in Biology and Biotechnology*. 2002;2:2-11.
- [27] Mackay IM. Real-time PCR in the microbiology laboratory. *Clinical Microbiology and Infection*. 2004;10:190-212.
- [28] Bustin SA, Benes V, Nolan T, Pfaffi MW. Quantitative real-time RT-PCR - a perspective. *Journal of Molecular Endocrinology*. 2005;34:597-601.
- [29] Houde A, Mattison K, Ward P, Plante D. Molecular detection and characterization of food and waterborne viruses. In Knudsen WD, Bruns SS, eds. *Bacterial DNA, DNA Polymerase and DNA Helicases*. NY, USA: Nova Science Publishers; 2009:137-185.
- [30] Centre Européen d'Expertise et de Recherche sur les Agents Microbiens (CEERAM). CEERAM Tools. Available at: <http://www.ceeramtools.com>. Accessed May 6, 2010.
- [31] Hasselback P. Feedlot alley and enteric illness – are they related or is Southern Alberta just a wonderful place for humans, cattle and bugs to live? Canadian Laboratory Medicine Congress, Calgary AB. May 2002.
- [32] Alberta Agriculture, Food and Rural Development. Census of Agriculture for Alberta: I.D., M.D., and County Data by Region. Agdex 852-1. 2001.
- [33] Ward P, Poitras E, Leblanc D, Letellier A, Brassard J, Plante D, Houde A. Comparative analysis of different TaqMan real-time RT-PCR assays for the detection of swine Hepatitis E virus and integration of Feline calicivirus as internal control. *Journal of Applied Microbiology*. 2009;106:1360-1369.
- [34] Mattison K, Brassard J, Gagné MJ, Ward P, Houde A, Lessard L, Simard C, Shukla A, Pagotto F, Jones TH, Trottier YL. The Feline calicivirus as sample process control for the detection of food and waterborne RNA viruses. *International Journal of Food Microbiology*. 2009;132:73-77.