J Vet Diagn Invest 8:241-244 (1996)

Use of nonradioactive cDNA probes to differentiate porcine respiratory coronavirus and transmissible gastroenteritis virus isolates

Eric M. Vaughn, Patrick G. Halbur, Prem S. Paul

Porcine respiratory coronavirus (PRCV), a member of the family Coronaviridae, is antigenically related to transmissible gastroenteritis virus (TGEV) of swine.[3,4,6,9,12,17,18,20,24] PRCV, now thought to be a mutant of TGEV,[3,9,13,23] was first isolated in 1984[12] from pigs in Belgium that were seropositive for TGEV but did not have a history of clinical transmissible gastroenteritis (TGE). Since the initial isolation of PRCV, it has been found that infections of swine in Europe with PRCV are widespread.[4,8,9] PRCV has also been isolated in swine in the United States,[7,11,20,24] but its prevalence in herds within the United States is not known.

There are several similarities between PRCV and TGEV. Both viruses have 3 major structural proteins: the surface spike (S) glycoprotein, the integral membrane glycoprotein, and an internal nucleoprotein.[3,9,13,24] Nucleotide sequences of PRCV isolates thus far studied show that they are closely related to TGEV[3,13,23] but that there are some striking differences. PRCV isolates have a characteristic deletion in the 5' end of the S gene when compared to TGEV,[3,8,13,20,23] and PRCV has a different tissue tropism than TGEV. TGEV replicates in both the respiratory and intestinal tissues and causes gastroenteritis,[14] whereas PRCV replicates to high titers in lung tissue of swine and with little or no replication in the intestinal tissues and no evidence of gastroenteritis and villous atrophy.[5,7,24]

PRCV is antigenically related to TGEV in that polyclonal sera which neutralize TGEV also neutralize PRCV.[4,6,12,17,24] Thus, conventional serologic methods are not useful in determining if a swine herd with anti-TGEV antibodies has been infected with PRCV or TGEV. Anti-TGEV neutralizing monoclonal antibodies (MAbs) directed against the S glycoprotein readily neutralize PRCV; however, there are some nonneutralizing anti-TGEV MAbs directed against the S glycoprotein that can be used to distinguish between PRCV and TGEV isolates in a competitive binding assay.[4,6,17,18]

Of the European PRCV isolates that have had their nucleotide sequences published, all have a 672-nucleotide deletion in the 5' end of the S gene.[3,13] The US PRCV isolates Ind/89 and ISU-1 have a 681-nucleotide deletion present in the 5' end of the S gene.[8,20,21,23] Recently the PRCV isolates AR310 and LEPP have been shown to have a smaller S gene deletion of 621 nucleotides present.[21] Additionally, the PRCV isolate IA1894 has recently been shown to have a 678-nucleotide deletion in the 5' end of its S gene.[21] Hence, a cDNA probe that encompasses the region of the TGEV S gene that is characteristically deleted from PRCV isolates can be used in a nucleic acid hybridization to differentiate between PRCV and TGEV isolates. We have developed two cDNA probes on the basis of the nucleotide sequence of the S gene of TGEV that can be used to differentiate between PRCV and TGEV isolates.[20] Other researchers have reported on the use of TGEV cDNA probes in a nucleic acid hybridization to differentiate between TGEV and PRCV isolates; however, these previously reported hybridization methods relied on using cDNA probes labeled with the radionuclide ^{32}P.[1,8,16,22] In this article we report on a nucleic acid hybridization assay that uses an immunochemiluminescent detection method to differentiate between TGEV and PRCV isolates, thus avoiding the use of radioactive materials.

The isolation of the PRCV isolates AR310, LEPP, and IA1894 has been previously described.[20] The PRCV isolates PON, IA725, and IA588 were isolated from nasal swabs from swine in herds that had antibodies to TGEV but no evidence of diarrhea and thus were suspected of having PRCV infection. Nasal swabs were collected, placed into 1 ml of minimum essential medium with 2% fetal bovine serum and antibiotics, and mixed for 20 seconds. Two hundred microliters of the nasal swab minimum essential medium was inoculated onto swine testicle (ST) cells. Cytopathic effect was present on the first passage in ST cells for the PRCV isolates PON, IA725, and IA588. The PRCV isolates AR310, LEPP, and IA1894 were plaque-purified a total of 3 times, and stock virus was stored at –70 C. The PRCV isolates PON, IA725, and IA588 were not plaque-purified for this study. All of the PRCV isolates replicated and produced CPE in the ST cell line on the first passage, and this appears to be characteristic of PRCV isolates.

The Miller strain of TGEV was used as the standard TGEV strain in this study. The PRCV isolate ISU-1 was received as a plaque-purified preparation and was kindly provided by Dr. Howard Hill (Iowa State University Veterinary Diagnostic Laboratory).[7]

ST cells were infected at a multiplicity of infection (moi) of approximately 0.1 plaque forming unit/cell with Miller TGEV and the PRCV isolates AR310, ISU-1, IA1894, or LEPP. At 19 hours postinfection, the medium was removed, and the total RNA was isolated from the infected ST cell monolayers by a rapid guanidinium thiocyanate method: The moi used for the PRCV isolates PON, IA725, and IA588 was unknown as these isolates were not plaque-purified and not titered. Total RNA from the ST cell monolayers infected with the PRCV isolates PON, IA725, and IA588 was isolated when the infected ST cell monolayers started showing approximately 25-30% CPE. Total RNA from uninfected ST cell monolayers was also isolated and used as a negative control. The RNA was washed with 70% ethanol, dissolved in diethyl pyrocarbonate-treated distilled water, and stored at -70 C.

Ten micrograms of total RNA from uninfected and TGEV-

From the Veterinary Medical Research Institute (Vaughn, Halbur, Paul) and the Department of Microbiology, Immunology, and Preventive Medicine (Vaughn, Paul), College of Veterinary Medicine, Iowa State University, Ames, IA 50011.

Received for publication March 16, 1995.

Downloaded from vdi.sagepub.com at Univ of Connecticut / Health Center / Library on May 23, 2015

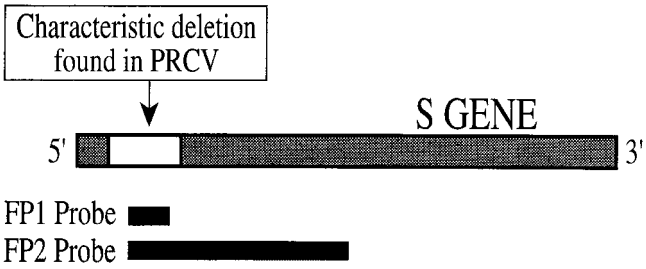

Figure 1. Schematic diagram of the S gene of TGEV indicating the characteristic deletion of PRCV and the location of the probes FP1 and FP2. The cDNA probe FP2 was used to detect the S gene of both TGEV and PRCV in a dot-blot hybridization assay, whereas the cDNA probe FP1 was used to detect only the S gene of TGEV in a dot-blot hybridization assay.

or PRCV-infected ST cell monolayers was denatured with formaldehyde and formamide[15] and then blotted to nylon membranes[b] using a 96-well hybridot manifold apparatus.[c] The membranes were washed in 10 x saline sodium citrate (SSC) (1 x SSC is 0.15 M NaCl plus 15 mM sodium citrate), allowed to air-dry, and then baked at 80 C for 2 hours to fix the RNA. The membranes were prehybridized for 2 hours in a solution containing 50% formamide, 5 x saline sodium phosphate EDTA (SSPE) (1 x SSPE is 0.18 M NaCl, 10 mM sodium phosphate, and 1 mM ethylenediaminetetraacetic acid [EDTA]), 4 x Denhardt's solution, 1.0% sodium dodecyl sulfate (SDS), and sonicated salmon sperm DNA (30 µg/ml) at 42 C. The cDNA probes used in the hybridization procedure are designated FP2 and FP1 (Fig. 1) and were described previously.[20] FP2 is a 2.28-kb polymerase chain reaction (PCR) product that was amplified by the primers 101004 (5' ggggatccAGAACTATAGGTAACCATTGG 3') and 060704 (5' ggggatccGCAGTGCCACGAGTCCTATCAT 3') and cut with the *Bam*HI restriction enzyme and cloned in the phagemid vector pKS+ .[d] FP1 is a 0.58-kb PCR product that was amplified by the primers 101004 and 100907 (5' gggggaaTTCTAATGTAGTCGCACGCAT 3') and cut with *Bam*HI and *Eco*RI restriction enzymes and cloned in the phagemid vector pKS+ .[d] FP2 encompasses the 5'-half of the TGEV S gene and thus will hybridize to both TGEV and PRCV S genes. However, FP1 encompasses the region of the TGEV S gene that is deleted from all PRCV isolates known thus far and will only hybridize with the S gene of TGEV. The FP1 and FP2 PCR products were originally amplified from a plasmid containing the Miller TGEV S gene using the appropriate primers under the following parameters: 1 cycle of 1 minute at 94 C, 1 minute at 48 C, and 5 minutes at 72 C; 30 cycles of 1 minute at 94 C, 1 minute at 48 C, and 3 minutes at 72 C; followed by 1 cycle of 1 minute at 94 C, 1 minute at 48 C, and 5 minutes at 72 C in a DNA thermal cycler.[e] To make cDNA probes the FP1 and FP2 PCR products were reamplified from their respective plasmids using the conditions described above. The FP1 and FP2 PCR products were then separated on a 2% low-melting-point agarose[f] gel and then purified from the agarose gel by a PCR purification kit.[g] Twenty-five nanograms each of FP1 and FP2 PCR products were labeled with digoxigenin-labeled deoxyuridine triphosphate in the presence of random hexamer primers and Klenow fragment of DNA polymerase I.[h] Unincorporated digoxigenin-labeled dUTP was removed by passing the labeled DNA through a Sephadex G-50 column.[h] The digoxigenin-labeled FP1 and FP2 probes were then heated to 100 C, cooled on ice, and then added to the appropriate prehybridization reaction and allowed to hybridize overnight. The membranes were washed once in 2 x SSC-0.3% SDS at room temperature, twice in 2 x SSC-0.3% SDS at 65 C, and once with 0.2 x SSC at 65 C. The membranes were washed in buffer 1 (100 mM maleic acid and 150 mM NaCl; pH, 7.5) for 1 minute, followed by a 30-minute incubation in buffer 2 (1% blocking agent[h]). The membranes were then incubated for 30 minutes in buffer 2 containing 150 mU/ml of alkaline-phosphatase-labeled anti-digoxigenin conjugate.[h] Unbound antibody conjugate was removed by washing the membranes twice in buffer 1 for 15 minutes. The membranes were equilibrated in buffer 3 (100 mM Tris-HCl, 100 mM NaCl, and 50 mM $MgC1_2$; pH, 9.5) for 2 minutes. For immunochemiluminescent detection, the membranes were repeatedly passed through the detection reagent[h] and enclosed in plastic wrap. Chemiluminescent signals were detected by exposing X-ray film[i] to the membranes. As expected, the probe FP2 detected the TGEV and PRCV mRNAs present in the total RNA isolated from the PRCV- and TGEV-infected ST cells (Fig. 2). The probe FP1 detected only TGEV mRNA present in the total RNA isolated from the TGEV-infected ST cells. The FP1 and FP2 probes did not hybridize to the RNA isolated from the uninfected ST cell culture.

The titers of PRCV AR310 and Miller TGEV were determined by plaque assay as previously described.[19] The PRCV isolate AR310 and the Miller strain of TGEV were diluted in minimum essential medium to a virus concentration of 10^6, 10^5, 10^4, 10^3, 10^2, or 10^1 PFU per 100 µl. SDS and proteinase K[j] were added to each dilution of PRCV or TGEV to a final concentration of 0.1% and 500 µg, respectively, mixed, incubated at 37 C for 30 minutes, and phenol/chloroform was extracted. The extracted viral RNA was then heated to 100 C for 5 minutes, cooled on ice, and blotted to nylon membranes as previously described.[10] The membranes were then used in a dot-blot hybridization assay as described above. Using RNA from virus-containing cell culture lysates showed that at least 10^5 PFU of TGEV could be detected (data not shown).

To determine the specificity of the nonradioactive probes, cell culture lysates from 2 group A rotaviruses (OSU and Gottfried), 3 enterovirus isolates (groups 1, 2, and 8c), and porcine parvovirus (NADL-2) were treated with SDS and proteinase K, boiled, and blotted to nylon membranes as previously described.[10] The membranes were then used in a dot-blot hybridization assay as described above. The FP1 and FP2 probes were shown to be specific as the probes showed no reactivity to the rotavirus, enterovirus, or parvovirus nucleic acids (data not shown).

The probe FP2 described in this study was specific for TGEV and PRCV RNA in an immunochemiluminescent dot-blot hybridization assay. Additionally, the probe FP1 was able to differentiate between PRCV and TGEV isolates an immunochemiluminescent dot-blot hybridization assay. This nucleic acid hybridization procedure takes advantage of the characteristic deletion in the 5' end of the S gene found

Downloaded from vdi.sagepub.com at Univ of Connecticut / Health Center / Library on May 23, 2015

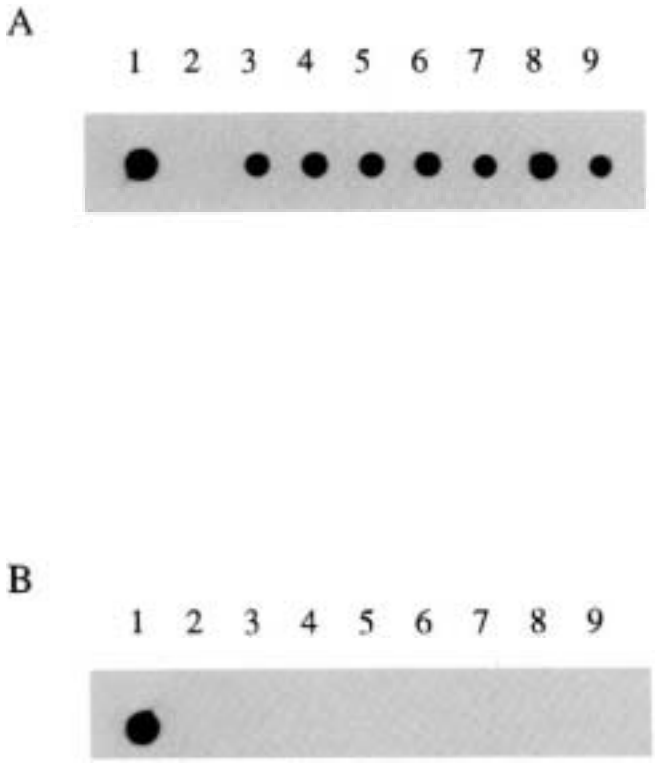

Figure 2. Dot-blot hybridization assay using the digoxigenin-labeled FP1 and FP2 cDNA probes and immunochemiluminescent detection of specific hybridization. **A.** The cDNA probe FP2 was labeled with digoxigenin-labeled dUTP and was hybridized with the total RNA from ST cell cultures that were infected with Miller TGEV (1), uninfected (2), or infected with the PRCV isolates ISU-1 (3), AR310 (4), IA1894 (5), LEPP (6), PON (7), IA725 (8), and IA588 (9). Note that the cDNA probe FP2 hybridized with total RNA from both TGEV- and PRCV-infected ST cell cultures. **B.** The cDNA probe FP1 was labeled with digoxigenin-labeled dUTP and was hybridized with the total RNA from ST cell cultures that were infected with Miller TGEV (1), uninfected (2), or infected with the PRCV isolates ISU- 1(3), AR310 (4), IA1894 (5), LEPP (6), PON (7), IA725 (8), and IA588 (9). Note that the cDNA probe FP1 hybridized only with total RNA from TGEV-infected ST cell cultures; the total RNA from PRCV-infected ST cell cultures was not detected with the cDNA probe FP1.

in PRCV isolates. All PRCV isolates studied thus far have had large deletions in the 5' end of the S gene ranging from 621 to 681 nucleotides in length.[3,8,13,20,21,23]

The source of TGEV or PRCV RNA to be used in the above described immunochemiluminescent dot-blot hybridization assay can be isolated in 1 of 2 ways. First, TGEV or PRCV RNA can be obtained from virus-containing lysates from infected cell cultures, and second, total RNA can be obtained from infected ST cell cultures that are displaying TGEV or PRCV CPE. When using RNA from virus-containing cell culture lysates, we found that at least 10^5 PFU of TGEV could be detected. Previously, it has been calculated that 2 x 10^6 virions of TGEV is equivalent to 25 pg of TGEV genomic RNA.[16] Thus, the 10^5 PFU detected in this study would indicate that the immunochemiluminescent dot-blot hybridization assay described here is able to detect the equivalent of 1.25 pg of TGEV genomic RNA. Other researchers have reported that TGEV-specific cDNA probes labeled with ^{32}P can detect from 25 to 200 pg of genomic TGEV RNA.[2,24] This indicates that the immunochemiluminescent dot-blot hybridization assay described here using digoxigenin-labeled cDNA probes is 20 to 160 times more sensitive than previous reports utilizing 32P-labeled cDNA probes.

Also, we have found that when using the immunochemiluminescent dot-blot hybridization assay procedure to detect TGEV or PRCV RNA in virus-containing cell lysates, a rather high background level was present. The high background generally was evident on exposure of the X-ray film to the nylon membranes for periods longer than 1 hour. Thus, it appears that isolating total RNA from infected ST cells that are showing CPE of PRCV or TGEV will give the best results in this procedure. This is most likely attributable to the ability of the probes to more readily detect the high copy numbers of genomic RNA and subgenomic mRNA 2 found in infected ST cells, as compared to single genomic RNA copies found in virions.

We have found that enterovirus contamination is a common occurrence in ST cell cultures when using nasal swabs to isolate PRCV from swine. Even though enterovirus causes a different CPE in ST cells than that produced by PRCV, enterovirus contamination should not be a problem in this assay as the cDNA probes used in this study were shown not to react to 3 different groups of enteroviruses (groups 1, 2, and 8c). As reported by other researchers, it would be expected that the probe FP2 would detect nucleic acid from antigenically related coronaviruses, such as feline infectious peritonitis virus and canine coronavirus.[1,2,22]

Although PRCV continues to be isolated from swine herds in the United States, its prevalence is not known. As more researchers attempt to obtain PRCV isolates for field studies on the prevalence of PRCV or to further study the molecular characteristics and pathogenicity of new PRCV isolates, the immunochemiluminescent dot-blot hybridization assay should be beneficial. The immunochemiluminescent dot-blot hybridization assay described in this article provides a rapid, specific, and nonradioactive detection system to differentiate PRCV from TGEV.

Acknowledgements. This study was supported in part by a grant from the Iowa Livestock Health Advisory Council. We thank Kelly Hicks for her excellent technical assistance.

Sources and manufacturers

a. Stratagene, La Jolla, CA.
b. Magna NT, Micron Separations Inc., Westboro, MA.
c. Schleicher and Schtill, Keene, NH.
d. Stratagene, La Jolla, CA.
e. Coy Corp., Grass Lake, MI.
f. NuSieve GTG, FMC Bioproducts, Rockland, ME.
g. Wizard PCR Prep, Promega, Madison, WI.
h. Genius 1 Kit, Boehringer Mannheim, Indianapolis, IN.
i. RX film, Fuji Photo Film Co., Stamford, CT.
j. Ameresco, Solon, OH.

References

1. Bae I, Jackwood DJ, Benfield DA, et al.: 1991, Differentiation of transmissible gastroenteritis virus from porcine respiratory

Downloaded from vdi.sagepub.com at Univ of Connecticut / Health Center / Library on May 23, 2015

coronavirus and other antigenically related coronaviruses by using cDNA probes specific for the 5' region of the S glycoprotein gene. J Clin Microbiol 29:215-218.
2. Benfield DA, Jackwood DJ, Bae I, et al.: 1992, Detection of transmissible gastroenteritis virus using cDNA probes. Arch Virol 116:91-106.
3. Britton P, Mawditt IU, Page KW: 1991, The cloning and sequencing of the virion protein genes from a British isolate of porcine respiratory coronavirus: comparison with transmissible gastroenteritis virus genes. Virus Res 21:181-198.
4. Callebaut P, Pensaert MB, Hooyberghs J: 1989, A competitive ELISA for the differentiation of serum antibodies from pigs infected with transmissible gastroenteritis virus (TGEV) or with the TGEV-related porcine respiratory coronavirus. Vet Microbiol 20:9-19.
5. Cox E, Hooyberghs J, Pensaert MB: 1990, Sites of replication of a porcine respiratory coronavirus related to transmissible gastroenteritis virus. Res Vet Sci 48: 165-169.
6. Garwes DJ, Stewart F, Cartwright SF, Brown, I: 1988, Differentiation of porcine coronavirus from transmissible gastroenteritis virus. Vet Rec 122:86-87.
7. Hill H, Biwer J, Woods R, Wesley R: 1990, Porcine respiratory coronavirus isolated from two U.S. swine herds. Proc Am Assoc Swine Pract, pp. 333-335.
8. Jackwood DJ, Bae I, Jackwood RJ, Saif LJ: 1992, Transmissible gastroenteritis virus and porcine respiratory coronavirus: molecular characterization of the S gene using cDNA probes and nucleotide sequence analysis. *In:* Coronaviruses: molecular biology and virus-host interactions, eds. Laude H, Vautherot JF, pp. 43-48. Plenum Press, New York, NY.
9. Laude H, van Reeth K, Pensaert MB: 1993, Porcine respiratory coronavirus: molecular features and virus-host interactions. Vet Res 24:125-150.
10. Meng XJ, Paul PS, Vaughn EM, Zimmerman JJ: 1993, Development of a radiolabeled nucleic acid probe for the detection of encephalomyocarditis virus of swine. J Vet Diagn Invest 5:254-258.
11. Paul PS, Vaughn EM, Halbur PG: 1992, Characterization and pathogenicity of a new porcine respiratory coronavirus strain AR310. Proc Int Pig Vet Soc Congr 12:92.
12. Pensaert M, Callebaut P, Vergote J: 1986, Isolation of a porcine respiratory, non-enteric coronavirus related to transmissible gastroenteritis. Vet Q 8:257-261.
13. Rasschaert D, Duarte M, Laude H: 1990, Porcine respiratory coronavirus differs from transmissible gastroenteritis virus by a few genomic deletions. J Gen Virol 71:2599-2607.
14. Saif LJ, Bohl EH: 1986, Transmissible gastroenteritis. *In:* Diseases of swine, eds. Leman AD, Glock RD, Mengeling WL, Penny RHC, Scholl E, Straw B, 6th ed., pp. 255-274. Iowa State University Press, Ames, IA.
15. Sambrook J, Fritsch EF, Maniatis T: 1989, Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
16. ShockIey LJ, Kapke PA, Lapps W, et al.: 1987, Diagnosis of porcine and bovine enteric coronavirus infections using cloned cDNA probes. J Clin Microbiol 25:1591-1596.
17. Simkins, RA, Weilnau PA, Van Cott J, et al.: 1993, Competitive ELISA, using monoclonal antibodies to the transmissible gastroenteritis virus (TGEV) S protein, for serologic differentiation of pigs infected with TGEV or porcine respiratory coronavirus. Am J Vet Res 54:254-259.
18. Van Nieuwstadt AP, Boonstra J: 1992, Comparison of the antibody response to transmissible gastroenteritis virus and porcine respiratory coronavirus, using monoclonal antibodies to antigenic sites A and X of the S glycoprotein. Am J Vet Res 53: 184-190.
19. Vaughn EM, Paul PS: 1993, Antigenic and biological diversity among transmissible gastroenteritis virus isolates of swine. Vet Microbiol 36:333-347.
20. Vaughn EM, Halbur PG, Paul PS: 1994, Three new isolates of porcine respiratory coronavirus with various pathogenicities and spike (S) gene deletions. J Clin Microbiol 32:1809-1812.
21. Vaughn EM, Halbur PG, Paul PS: 1995, Sequence comparison of porcine respiratory coronavirus isolates reveals heterogeneity in the S, 3, and 3-1 genes. J Virol 69:3176-3184.
22. Wesley RD, Wesley IV, Woods RD: 1991, Differentiation between transmissible gastroenteritis virus and porcine respiratory coronavirus using a cDNA probe. J Vet Diagn Invest 3:29-32.
23. Wesley RD, Woods RD, Cheung AK: 1991, Genetic analysis of porcine respiratory coronavirus, an attenuated variant of transmissible gastroenteritis virus. J Virol 65:3369-3373.
24. Wesley RD, Woods RD, Hill HT, Biwer JD: 1990, Evidence for a porcine respiratory coronavirus, antigenically similar to transmissible gastroenteritis virus, in the United States. J Vet Diagn Invest 2:312-317.

Downloaded from vdi.sagepub.com at Univ of Connecticut / Health Center / Library on May 23, 2015