PEER REVIEWED

The Survival of Bovine Viral Diarrhea Virus on Materials Associated with Livestock Production

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Abstract

Fomites have been shown to play a role in the transmission of bovine viral diarrhea virus (BVDV). However, there are no published reports on the length of time BVDV can survive on fomites. In the current study, we applied a type 1b, non-cytopathic (NCP) BVDV isolate to clothing, materials common to livestock production settings, and various feedstuffs. Additionally, we created a synthetic mucus phosphate buffered saline (PBS) solution and added BVDV to determine whether mucus impacted BVDV survival. Overall, the ability to recover BVDV after application to potential fomites decreased as the length of incubation increased. The BVDV strain tested in this study exhibited longer survival times in two aqueous solutions (water and PBS), two non-porous materials (latex and enameled metal), and one porous material (paper) than in other potential fomites. One non-porous material (galvanized metal) and two porous materials (soil and pine) tended to have a lower chance of BVDV recovery. No virus was recovered from denim, cotton t-shirt, mineral and salt blocks. For the total mixed ration, BVDV was recovered in one replicate, for up to eight hours post-application; however, all other replicates were negative. The molasses-urea lick in all three replicates was contaminated, therefore they were not tested. Based on these findings, a type 1b, NCP BVDV was capable of surviving after application to various materials used in livestock production. In the presence of mucus, BVDV was protected from degradation for longer periods of time.

Keywords: bovine, fomite, mucus, BVDV, pestivirus survival

Résumé

Il a été démontré que les vecteurs passifs jouaient un rôle dans la transmission du virus de la diarrhée virale des bovins (BVD). Toutefois, aucune étude publiée n'indique combien de temps le virus du BVD survit sur ces vecteurs passifs. Dans la présente étude, nous avons appliqué un isolat non cytopathogène (ncp) de type 1b du virus du BVD sur des vêtements, du matériel et des produits alimentaires couramment utilisés dans les élevages de bétail. De plus, nous avons inoculé cette souche de virus du BVD dans un mucus synthétique à base de solution saline dans un tampon phosphate (PBS) que nous avons préparé, pour déterminer si le mucus influence la survie de ce virus. Dans l'ensemble, notre capacité á détecter des particules vivantes de cette souche du virus du BVD sur les vecteurs passifs a diminué avec la durée d'incubation. Dans deux solutions aqueuses (eau et PBS), sur deux matériaux non poreux (latex et métal émaillé) et sur un matériau poreux (papier), la souche du virus de BVD employée dans cette étude a survécu plus longtemps que sur d'autres matériaux de vecteurs passifs potentiels. Sur un matériau non poreux (métal galvanisé) et deux matériaux poreux (terre et planches de pin), nous avons prélevé moins de particules vivantes de vírus du BVD. Nous n'avons pas trouvé de particules vivantes de la souche étudiée sur le denim, les t-shirts en coton, les minéraux et les blocs de sel. Dans la ration totale mélangée, nous avons observé ce virus vivant jusqu'à huit heures après l'application dans l'une des répétitions, mais aucun dans les autres répétitions. Les blocs de mélasse à l'urée étaient contaminés dans la totalité des trois répétitions Ces résultats nous permettent de dire que la souche de virus du BVD

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non cytopathogène de type 1b peut survivre après son application sur divers matériaux utilisés en production bovine. De plus, la présence de mucus a prolongé la survie de cette souche de virus du BVD en freinant sa dégradation.

Introduction

The manifestations of bovine viral diarrhea virus (BVDV) are varied and of economical concern to beef and dairy producers at all production levels. BVDV is an enveloped, single-strand RNA virus in the genus *Pestivirus* that may cause a number of clinical outcomes. Even without apparent clinical signs, acutely infected cattle shed BVDV in nasal secretions and other body fluids.[5] Another important reservoir for BVDV is persistently infected (PI) BVDV animals. Whether from an acute or persistent infection, animals shedding BVDV are the primary source of infection.

Fomites have been shown to transmit BVDV. A fomite is an object that may be contaminated with infectious organisms and serves to transmit pathogens to susceptible animals. In one study, researchers rectally palpated a PI-BVDV animal and then immediately rectally palpated naïve heifers without changing rectal examination sleeves. All animals rectally palpated developed antibodies to BVDV (eight of eight animals), and more than half of the same animals were viremic (five of eight animals).[4] In another study, researchers demonstrated that hypodermic needles and nose tongs could transmit BVDV to naïve animals.[3] Additionally, non-biting flies, which fed on ocular secretions, were also contaminated with BVDV.[3]

Studies have shown that many human viruses, including poliomyelitis virus, rotavirus, hepatitis virus, and adenovirus are capable of surviving outside the host for as long as 60 days.[10] Survival of BVDV in the environment may be important to its ability to spread.[11] It has been reported that both BVDV biotypes, cytopathic and non-cytopathic, rapidly lose infectivity after contact with organic solvents or pH outside a range of 5.7 to 9.3. BVDV survival decreases in low pH as environmental temperature increases from 39.2°F to 98.6°F (4°C to 37°C).[2,6] Classic swine fever virus, another pestivirus, is unstable at low or high pH, or high temperatures, and does not persist in the environment for more than two weeks.[6]

Although research has demonstrated BVDV can be transmitted by a fomite, there are no published studies on the longitudinal survival of BVDV on common materials and within liquids used in livestock production that could serve as fomites. The objective of this study was to evaluate the longitudinal survival of a type 1b, non-cytopathic BVDV on fomites in the presence or absence of a novel, synthetic mucus.

Materials and Methods

Virus

The BVDV strain CA0401186a was used for testing survival on fomites.[a] This strain was isolated from the tissues of a persistently infected calf submitted to the National Animal Disease Center (NADC) by diagnosticians from the California Animal Health and Food Safety Laboratory, Tulare CA. The calf was one of 24 premature calves with brain and skeletal deformities born to heifers in one herd. CA0401186 is a non-cytopathic BVDV 1b.[8]

Preparation of Viral Suspensions

The virus was propagated in BVDV-free bovine turbinate (BT) cells to produce a single lot of virus for the study. After propagation, the virus was aliquoted, frozen, and stored at –112°F (–80°C) for future use. Two vials of virus, the first and last aliquot of the lot, were used for determination of the titer. Based on the final titer, a working stock of virus for the experiment was created at 4.1 $\log_{10}$ median tissue culture infective dose ($TCID_{50}$)/mL, which was diluted in either phosphate buffered saline[b] (PBS; pH=7.4) or a 20% mucus/PBS solution (pH = 7.4). The amount of virus applied to or into each material was normalized so that all materials started with the same amount of virus, regardless of the final volume applied to the respective material.

Preparation of Mucus

Synthetic mucus was prepared according to a previously published protocol.[1] It contained 1 g guar gum, 0.5 g dried type II mucin from porcine stomach, 0.26 g monobasic potassium phosphate, 1.57 g dibasic potassium phosphate, 1 mL 0.1 M sodium borate solution, and 96.2 g distilled water.[c] The mixing process involved slow dissolution of guar gum into 90% of the distilled water using a standard magnetic stir bar. After complete hydration, noted by a visual increase of the solution's viscosity, mucin was slowly added. While the mucin mixed, the buffer salts were dissolved into the remaining 10% of the distilled water and then slowly added to the viscous solution. Once mixed well, the borate solution was added to the solution. The synthetic mucus was then sterilized by autoclaving prior to use in the study.

Preparation and Inoculation of Fomite Samples

Paper (computer print paper), latex glove material, and clothing (Hanes T-shirt or denim jeans)

One-cm squares were cut from each of the materials. They were sterilized by autoclaving, and after drying, each piece was placed in a separate well of a 24-well culture plate.[d] A total of 40 µl, in two separate 20-µl aliquots, of the virus suspension was added to each fomite.

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Wood (untreated pine), rubber (Wellington boot), metal (galvanized or enameled steel)

One-cm squares were cut, autoclaved and allowed to dry. Each fomite was placed in a separate well of a six-well culture plate.[d] A total of 40 µl, in two separate 20-µl aliquots, of the virus suspension was added to each fomite.

Mineral, Salt, and Molasses Urea Blocks

An approximate 0.5 – 1.0 cubic centimeter piece of mineral, salt, or of a molasses urea lick were obtained from the block or lick using a hammer and chisel. A total of 40 µl, in two separate 20-µl aliquots, of the virus suspension was added to each fomite.

Total Mixed Ration and Feedlot Pen Soil

Aliquots of 0.3 g of each material were weighed, placed into a 2.0-mL centrifuge tube,[e] autoclaved, and allowed to dry prior to use. A total of 40 µl, in one aliquot, of the virus suspension was added to each fomite.

Water & Phosphate Buffered Saline (PBS)

Distilled water and PBS[b] were autoclaved. After autoclaving, 40 µl of the virus suspension was added to each fomite.

Collection of Samples

Samples for virus isolation were collected immediately after the virus suspension was applied to the fomite (0 hour). Additionally, samples were collected at 1, 2, 4, 6, 8, 24, 48, and 96 hours. Fomites were eluted with 1.0 mL (paper, latex, cotton t-shirt, denim, rubber, metal (galvanized and enameled), wood, mineral block, salt block, molasses urea lick) or 1.5 mL (feed and dirt) of virus maintenance media. Virus maintenance media (VMM) contained Minimum Essential Medium Eagle (MEM) supplemented with Earle's Salts,[c] 2% horse serum,[f] 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B,[f] and 2 mM L-glutamine.[f]

Virus Detection

Virus isolation in BT cells was attempted on every sample. The propagation and detection has been described previously.[9] Briefly, BT cells were seeded two days prior to use into a 96-well cell culture microtiter plate.[d] A total of 100 µl of the eluted sample was added to each well in triplicate. After plating all fomite samples, 100 µl of virus maintenance media was added to all wells. After four days of incubation at 98.6°F (37°C) in 5 % CO_2, the plates were frozen at –112°F (–80°C) and thawed three consecutive times. After the last freeze-thaw, 100 µl of the supernatant for each respective sample and 100 µl of virus maintenance media was transferred to another 96-well cell culture microtiter plate seeded with BT cells as described above. This was repeated one additional time, resulting in three passages for virus isolation. After the third pass, the media was removed and the plates were dried at room temperature. After thoroughly drying, the monolayers were fixed in a 20% acetone PBS solution for 10 minutes and then allowed to air dry. The fixed monolayers were incubated with a 1:400 dilution of the BVDV monoclonal antibody 20.10.6 in PBS containing 0.05% Tween 20[g] for 45 minutes at 98.6°F. Following four washes with PBS containing 0.05% Tween 20, the plates were incubated with a 1:400 dilution of biotinylated rabbit anti-mouse IgG[h] in binding buffer supplemented with 60% chicken serum[f] for 45 minutes at 98.6°F. Following a wash step as above, a 1:1000 dilution of peroxidase-conjugated streptavidin[i] was added and the plate was incubated for 45 minutes at 98.6°F. After a final wash, substrate containing 280 µg of 3-amino-9-ethylcarbazole[i] per mL and 0.01% hydrogen peroxide in 50 mM acetate buffer (pH 5.0) was added and the mixture was incubated at 98.6°F in the dark. A positive reaction was recorded when there was an appearance of a red intracellular precipitate after 30 minutes. Test samples were positive if they produced a distinct cytoplasmic staining in at least one well. If no color development was observed in the three wells, the sample was deemed negative.

Data Analysis

Data were analyzed using the logistic procedure in STATA 10.1™.[j] The data was transformed to model the effect of time on the likelihood of recovering virus at each time point using logistic regression. To achieve model stability, all of the 96-hour time-point observations were dropped because of lack of variability in the data (predicted no virus present perfectly). Also, observations for fomites cotton t-shirt, denim, total mixed ration (TMR), mineral lick, salt lick, and molasses-urea lick were dropped, as they perfectly predicted that no virus was found. Therefore, the logistic model was derived from a total of 486 observations. Due to the inherent study design, only the first-order model was explored. Therefore, no interactions were statistically analyzed. After achieving a satisfactory model, the PRVALUE procedure in STATA™ was used to achieve model adjusted risk estimates and corresponding 95% confidence intervals for the risk of finding virus present at each time point for each fomite (metal, wood, rubber, etc...) and treatment (PBS and mucus).

Results

Data for the cotton t-shirt, denim, TMR, mineral lick, salt lick, and molasses-urea lick were not included in the final analysis. No virus was recovered from the denim and cotton t-shirt elutions. For the TMR, for one replicate, we recovered BVDV for up to eight hours

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post-application; however, all other replicates were negative. The molasses-urea lick in all three replicates was contaminated, which resulted in a no-test. Lastly, the mineral and salt licks were negative at all time points. Overall, recovery of BVDV decreased as the length of incubation increased for other potential fomites, including in both mucus viral suspension (MV) and PBS viral suspension (PV).

Porous Materials - Paper, Soil, and Pine

On paper, BVDV tended to be recovered for longer periods of time in mucus than PBS (Table 1; $P = 0.052$). There were significant differences between MV and PV at two, four, and six hours post-incubation. Characterization of BVDV survival demonstrated there was a 93.7% chance that BVDV would be recovered at one hour for MV and a 62.1% chance that BVDV would be recovered at one hour for PV. At six hours post-incubation, there was only a 39.1% chance that BVDV would be recovered in the MV group compared to 6.6% in the PV group. By 48 hours post-incubation, there was less than 7% chance that BVDV would be present in either group.

As compared to other fomites, the survival of BVDV was reduced when applied to soil (Table 1). Characterization of survival demonstrated there was a 47.9% chance that BVDV would be recovered at one hour for MV and a 9.2% chance that BVDV would be recovered at one hour for PV. After eight hours, there was less than 2% chance of the virus being recovered from the soil.

The survival of BVDV was reduced when applied to untreated pine (Table 1). Characterization of survival demonstrated a 31.1% chance that BVDV would be recovered at one hour for MV and a 4.8% chance that BVDV would be recovered at one hour for PV. After eight hours, there was less than 1% chance of the BVDV being recovered from the untreated pine.

Non-porous: Latex, Rubber, Enameled and Galvanized Metals

With latex, BVDV tended to be recovered for longer periods of time in mucus than PBS (Table 1; $P = 0.08$). Significant differences between MV and PV were found at four and six hours post-incubation. Characterization of survival demonstrated there was a 98.2% chance that

Table 1. Model adjusted probability estimate of each fomite testing positive for BVDV strain CA0401186a after application to the potential fomite. Prior to application to the potential fomite, the virus was diluted in PBS or a PBS that included 20% (w:v) synthetic mucus. After application of BVDV to the potential fomite, the fomite was rinsed with PBS and the harvested PBS was tested to determine the presence or absence of BVDV with a three-pass virus isolation technique.

		Time (hrs) →							
Fomite	**Treatment**	**1**	**2**	**4**	**6**	**8**	**12**	**24**	**48**
Paper	PBS	62.10%	38.7% a	19.6% b	6.6% c	3.10%	2.40%	1.20%	0.80%
	Mucus	93.70%	85.0% a	68.7% b	39.1% c	22.10%	18.00%	10.20%	6.60%
Latex	PBS	85.80%	70.00%	47.4% d	20.8% e	10.40%	8.30%	4.50%	2.80%
	Mucus	98.20%	95.50%	89.1% d	70.4% e	51.20%	44.80%	29.60%	20.70%
Rubber	PBS	46.3% f	24.9% g	11.4% h	3.60%	1.60%	1.30%	0.70%	0.40%
	Mucus	88.6% f	75.0% g	53.6% h	25.20%	13.00%	10.40%	5.60%	3.60%
Pine	PBS	4.80%	1.90%	0.70%	0.20%	0.10%	0.10%	0.00%	0.00%
	Mucus	31.10%	14.80%	6.30%	1.90%	0.90%	0.70%	0.40%	0.20%
Galvanized metal	PBS	3.00%	1.20%	0.50%	0.10%	0.10%	0.10%	0.00%	0.00%
	Mucus	21.50%	9.60%	3.90%	1.20%	0.50%	0.40%	0.20%	0.10%
Enameled metal	PBS	46.3% i	24.9% j	11.4% k	3.60%	1.60%	1.30%	0.70%	0.40%
	Mucus	88.6% i	75.0% j	53.6% k	25.20%	13.00%	10.40%	5.60%	3.60%
Soil	PBS	9.20%	3.80%	1.50%	0.40%	0.20%	0.20%	0.10%	0.10%
	Mucus	47.90%	26.20%	12.00%	3.80%	1.70%	1.40%	0.70%	0.40%
PBS	PBS	65.60%	42.3% l	22.1% m	7.60%	3.50%	2.80%	1.40%	0.90%
	Mucus	94.50%	86.9% l	71.9% m	42.70%	24.80%	20.30%	11.70%	7.60%
Water	PBS	82.00%	63.8% n	40.4% o	16.50%	8.10%	6.40%	3.40%	2.10%
	Mucus	97.60%	94.1% n	86.0% o	64.10%	44.20%	37.90%	24.00%	16.40%

*Significant differences ($P < 0.05$) between treatments (PBS/mucus) within a given time point (column) for each fomite are noted by identical letters (a, a).

**Significant differences ($P < 0.05$) between time points within each treatment (PBS/mucus) for each fomite are noted with differing letters (a, b, c).

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BVDV would be recovered at one hour for the MV and an 85.8% chance that BVDV would be recovered at one hour for PV. At six hours post-incubation, there was a 70.4% chance that BVDV would be recovered in MV compared to 20.8% in PV. At 48 hours, there was a 20.7% chance that BVDV would be recovered in the MV group and less than 3% in the PV group.

With rubber excised from a Wellington boot, BVDV tended to be recovered for longer periods of time in mucus than PBS (Table 1; $P = 0.06$). Significant differences between MV and PV were demonstrated at one, two, and four hours post-incubation. Characterization of survival demonstrated there was an 88.6% chance that BVDV would be recovered at one hour for MV and a 46.3% chance that BVDV would be recovered at one hour for PV. At six hours post-incubation, there was a 25.2% chance that BVDV would be recovered in MV compared to 3.6% in the PV. By 48 hours post-incubation, there was less than 4% chance of survival from either group.

On enameled metal, BVDV survived longer in mucus than PBS (Table 1; $P < 0.05$). Significant differences between MV and PV were found at one, two, and four hours post-incubation. There was an 88.6% chance that BVDV would be recovered at one hour for MV and a 46.3% chance that BVDV would be recovered at one hour for PV. At six hours post-incubation, there was a 25.2% chance that BVDV would be recovered in the MV group compared to 3.6% in the PV group. At 48 hours post-incubation, there was less than 4% chance of recovering BVDV from either group.

Overall, the recovery of BVDV was reduced when BVDV was applied to galvanized metal (Table 1). Characterization of survival demonstrated there was a 21.5% chance that BVDV would be recovered at one hour for MV and a 3.0% chance that BVDV would be recovered at one hour for PV. At and after eight hours, there was less than 1% chance of BVDV being recovered.

Aqueous: Phosphate Buffered Saline (PBS) and Distilled Water

When PBS was supplemented with BVDV it tended to be recovered for longer periods of time in mucus (Table 1; $P = 0.052$). Significant differences between MV and PV were found at two and four hours post-incubation. Characterization of survival demonstrated there was a 94.5% chance that BVDV would be recovered at one hour for MV and a 65.6% chance that BVDV would be recovered at one hour for PV. At six hours post-incubation, there was a 42.7% chance that BVDV would be recovered in the MV group compared to less than 7.6% in PV. By 48 hours post-incubation, there was less than 8% chance of recovery from either group.

When distilled water was supplemented with BVDV, it was generally recovered for longer periods of time in mucus than PBS (Table 1). There were significant differences between MV and PV at four and six hours post-incubation. Characterization of survival demonstrated a 97.6% chance that BVDV would be recovered at one hour for MV and an 82.0% chance that BVDV would be recovered at one hour for PV. At six hours post-incubation, there was a 64.1% chance that BVDV would be recovered in MV compared to 16.5% in the PV. At 48 hours post-incubation, there was a 16.4% chance that BVDV would be recovered in MV compared to 2.1% in PV.

Discussion

Overall, the ability to recover BVDV after application to potential fomites decreased as the length of incubation increased. The BVDV strain we tested exhibited longer survival times in two aqueous solutions (water and PBS), two non-porous materials (latex and enameled metal), and one porous material (paper) than other potential fomites. One non-porous material (galvanized metal) and two porous materials (soil and pine) tended to have the lowest chance of recovering BVDV. Based on these findings, a type 1b, NCP BVDV was capable of surviving after application to various materials used in livestock production. When in the presence of mucus, BVDV appeared to survive for longer periods of time.

Based on our observations, the absorbency of the materials may have an effect on the survival of BVDV. When controlling for treatment, there was a numerical difference in the survival between non-absorbent materials (enameled metal, rubber boot, and latex glove) and absorbent materials (paper, cotton, soil, and pine). Additionally, the thickness of the pine may have negatively impacted the recovery of BVDV. When the thickness of the pine is compared to that of paper, which had a higher chance of recovery than pine, it is probable that the elution volume was better suited to penetrating paper than the pine and allowed for optimal recovery of BVDV.

Bovine viral diarrhea virus was recovered for longer periods of time when applied to latex using either PBS or water. When BVDV was in the presence of mucus, it was recovered for longer periods of time in water. This is a notable finding when considering livestock watering systems, since the systems generally provide water to a number of cattle at any one time. Therefore, if a PI-BVDV animal or an acutely infected BVDV animal was using a shared water source, it is conceivable that BVDV could persist in the water source and serve as a reservoir of BVDV for naïve cattle.

One interesting finding was the result obtained from galvanized metal. The importance of this result is unclear. Galvanization is a metallurgical process that is used to coat steel or iron with zinc to prevent rust. The galvanized metal was washed and autoclaved prior to use, so if the manufacturing process had introduced

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noxious chemicals they potentially would have been removed prior to our use. Zinc in the form of zinc oxide is commonly added to nursery pig diets to alleviate diarrhea and improve performance of nursery pigs.[12] The mode of action of ZnO has yet to be determined; however, the antimicrobial properties of Zn are well recognized in human medicine.[7] Therefore, based on our observations, it is conceivable that galvanized metal may inhibit the survival of BVDV by an unknown mode of action associated with zinc.

Data for the cotton t-shirt, denim, TMR, mineral lick, salt lick, and molasses-urea lick were not included in the final analysis. No BVDV was recovered from the denim and cotton t-shirt elutions. For one replicate, we recovered virus from the TMR for up to eight hours post-application, however, all other replicates were negative. All three replicates of the molasses-urea lick was contaminated, which invalidated the analysis. Lastly, the mineral and salt licks were negative at all time points. Interestingly, after mineral and salt-lick elutions were applied to BT cells, the monolayers were confluent and appeared normal. One possible reason for the negative results could be a change in the osmolality in the cell culture media. Dialysis of the elutions prior to plating may have been beneficial to remove the salts prior to testing.

A shortfall of the current study is that it was completed in a controlled environment with defined conditions using an *in vitro* system. We chose the controlled environment because it would have been impossible to mimic all of the diverse environmental conditions that could impact BVDV survival; however this study provides a starting point for assessment of BVDV survival. Another shortfall of this study is that we do not know if the BVDV recovered from a fomite can induce clinical or subclinical disease in naïve animals. However, we chose to use three-pass virus isolation for our propagation system as opposed to using polymerase chain reaction (PCR) on the eluates, which allowed us to determine that the virus we recovered was indeed viable.

Based on the findings of this *in vitro* study, the addition of a biosecurity plan for working with cattle is important and should be tailored accordingly. The practice of washing boots, changing gloves, disinfecting livestock chutes and other equipment, and cleaning water tanks are important mechanisms that may mitigate the risk of BVDV transfer from fomites.

Conclusions

Under the conditions of this study, a NCP, type 1b, BVDV was capable of surviving after application to various materials used in livestock production. When in the presence of mucus, BVDV was protected from degradation for longer periods of time than when not in the presence of mucus.

Endnotes

[a]The CA0401186 BVDV strain was a kind gift from Dr. Julia Ridpath (National Animal Disease Center, Ames, IA).
[b]Phosphate buffered saline, Cellgro, Manassas, VA
[c]Chemicals were purchased from Sigma-Aldrich, St. Louis, MO
[d]Greiner Bio-One North America, Monroe, NC
[e]Continental Lab Products, San Diego, CA
[f]Atlanta Biologicals, Lawrenceville, GA
[g]Sigma Aldrich, St. Louis, MO
[h]Invitrogen, Carlsbad, CA
[i]Invitrogen, Carlsbad, CA
[j]College Station, TX

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