#### ABSTRACT

#### CAROLINE G. COLDEN

An Immunohistochemical Study of the Temporal Distribution of the Vesicular Stomatitis Virus and the Preferred Cells for Viral Replication in Experimentally Infected Cattle

Under the Direction of DR. CORRIE BROWN

Vesicular stomatitis virus (VSV) is a single-stranded, negative-sense arbovirus in the Family Rhabdoviridae. Cattle, pigs, and horses can become infected and the virus settles in and causes damage to the surface tissues of the feet, tongue, snout, and teats, causing vesicular (blistering) lesions. Infection can have debilitating effects on the animals, as they are reluctant to eat, nurse, or move around. They lose a great deal of weight, which diminishes their economic value. In order to devise effective control measures, it is important to understand how the virus spreads around the body and how it damages cells. In this study, immunohistochemistry was used to detect the presence of the virus in tissues of cattle experimentally infected with VSV. Virus was inoculated into the skin at the top of the hoof (coronary band) and tissues were examined sequentially to envision presence of virus and associated damage. By immunohistochemistry, VSV was present in the coronary bands 12, 24, 48, 72, 96, and 120 hours post-infection (hpi) with highest intensity in the coronary bands at 48 and 72hpi. Virus appeared predominantly in cells of the stratum spinosum layer, which exhibit unique intercellular bridges. Special staining for these intercellular bridges revealed a marked correlation between presence of this structure and replicating virus, indicating that the intercellular bridges may be functioning in viral entry or transport.

INDEX WORDS: Vesicular Stomatitis, Immunohistochemistry, Cattle, Epidermis, Desmogleins

## AN IMMUNOHISTOCHEMICAL STUDY OF THE TEMPORAL DISTRIBUTION OF THE VESICULAR STOMATITIS VIRUS AND THE PREFERRED CELLS FOR VIRAL REPLICATION IN EXPERIMENTALLY INFECTED CATTLE

by

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in Partial Fulfillment of the Requirements for the Degree

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# AN IMMUNOHISTOCHEMICAL STUDY OF THE TEMPORAL DISTRIBUTION OF THE VESICULAR STOMATITIS VIRUS AND THE PREFERRED CELLS FOR VIRAL

## REPLICATION IN EXPERIMENTALLY INFECTED CATTLE

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## CHAPTER 1: INTRODUCTION

Vesicular Stomatitis is a disease caused by the Vesicular Stomatitis Virus (VSV). The large, bullet-shaped virus is a member of the genus *Vesiculovirus* and family Rhabdoviridae, and has a linear, single-stranded, negative-sense RNA genome that follows a replication cycle representative of most RNA viruses.<sup>1,3</sup> Its virion (65-185nm) is composed of a host-derived plasma membrane, an envelope, and an internal ribonucleoprotein core, the entire virion structure containing only five proteins.<sup>3,6</sup> VSV is categorized into two major serotypes: VSNJV (New Jersey strain) and VSIV (Indiana strain). Although both strains have been found in the US, they are usually not present in livestock and so are regarded as "foreign" when they appear in cattle or pigs, and control programs are undertaken to eradicate them.<sup>1,3,6</sup>

Vesicular Stomatitis virus infects livestock primarily, but is also capable of infecting humans, and the virus is believed to transmit itself through insect vectors.<sup>1,2,3,6</sup> Clinical signsof infection in livestock include the formation of painful blisters (vesicles) on the coronary bands, lips, gums, tongue, nostrils, hooves, or teats, depending on the animal host.<sup>1,3,6</sup> Infection is usually accompanied by fever and a host inflammatory response, as well as excessive salivation if the lesions occur in the mouth. Vesicular Stomatitis is not fatal, though, and recovery time is approximately two weeks if no secondary infections or complications arise. Infection in humans is rare and usually happens only in laboratory situations where there is heavy exposure. Humans develop influenza-like symptoms upon infection with VSV, which can often go misdiagnosed as

the fever, muscle aches, nausea, and malaise represent common symptoms of many other diseases.<sup>1,3,6</sup>

Lesions occur in the epidermis of infected animals, which is the outermost layer of skin, derived from the ectoderm during development, and is specifically composed of keratinized stratified squamous epithelial tissue.<sup>2,6,7</sup> The vast majority (85%) of cells that comprise the epidermis are known as keratinocytes, which are organized into epidermal layers according to their morphology and degree of differentiation.<sup>7</sup> Cells differentiate as they undergo a process called keratinization, by which the nuclei and cytoplasmic organelles gradually disintegrate and are replaced by a rich mixture of the protein keratin and other fibrous, durable substances (keratohyalin). The basal layer, or stratum basale, is the deepest layer of the epidermis, resting on top of the basement membrane (aids in epithelial cell movement and exchange with capillaries); its single layer of cells is the most undifferentiated, containing prominent nuclei, and serve as an anchor for the epidermis and reservoir of stem cells that will later proliferate and differentiate into gradually more keratinized cells that provide the outer layer of skin with its protective properties. The stratum spinosum (or "prickle" layer) rests on top of the stratum basale, and is characterized mainly by the presence of intercellular bridges or junctions (i.e., desmosomes) that bind cells tightly together.<sup>7</sup> It is for this reason the stratum spinosum is so named, as these intercellular processes confer a distinctive, "spiny" look to the cells when viewed microscopically.<sup>7</sup> The stratum granulosum lies above the stratum spinosum, but it is not present in all stratified squamous epithelia, such as the mucous membranes of the mouth, or is very thin, such as in neck or flank skin. The stratum lucidum, a thin, clear layer of cells between the stratum granulosum and topmost layer of epidermis, stratum corneum, is present only in regions where skin is particularly thick or hairless. Most often, however, only the stratum corneum is

present, which composes the outermost layer of the epidermis, with cells that are completely keratinized and devoid of nuclei or cytoplasmic organelles (and are thus dead as a result), and coated with a lipid matrix to form a "brick and mortar" organization.<sup>7</sup> These cells are constantly shed and newly keratinized cells replace them and resume their role of protecting the body against fluid loss, environmental damage, and infection.<sup>7</sup>

The intercellular bridges, known as desmosomes that characterize the epidermal layers, most notably the stratum spinosum, consist of several plaque proteins and core glycoprotein components.<sup>4,5,7</sup> Desmosomes help the epidermis resist mechanical stress by forming a meshwork system that connects keratin intermediate filaments of adjacent cells (keratinocytes) and their cytoskeletons.<sup>4,5</sup> There are several types of adhesion molecules that form the intercellular "bridge" structures (as they appear under light microscopes), for example the desmoglein family. Desmogleins are found throughout the more differentiated layers of the epidermis, including the strata spinosum, granulosum, and corneum, where desmosomes are prominent and serve to preserve mechanical integrity of the epithelial tissue.<sup>4,5,7</sup>

The transmission of VSV has not been completely elucidated, though insect vectors, such as sandflies, black flies, and mosquitoes, are believed to play a prominent role.<sup>1,2,3,6</sup> Direct contact between animals, or with contaminated fomites, as animals shed virus through saliva or fluid from ruptured vesicles is also thought to be a major mode of transmission as spread within herds can be rapid once VSV has been introduced.<sup>1,3,6</sup> However, the cellular receptor that allows for VSV entrance into the body has not yet been elucidated. Humans can become infected by VSV through contact with infected animals or fomites, insect bites, or by aerosol transmission (in laboratories).<sup>1,3</sup>

Vesicular Stomatitis causes considerable problems for the livestock industry in the warmer regions of the American continents, infecting cattle, horses, swine, and other livestock seasonally each year, with attack rates as high as 96%.<sup>1,2,3,6</sup> The virus is virtually indistinguishable clinically from several other diseases including foot-and-mouth disease, swine vesicular disease, and vesicular exanthema of swine that cause similar, debilitating symptoms.<sup>1,2,3,6</sup> The United States' exportation of livestock and animal products could suffer greatly as a result of restrictions enacted to prevent the spread of VSV to non-infected countries, thus rendering proper diagnosis of VSV essential to the containment and management of outbreaks.<sup>1</sup>

Laboratory analysis is crucial for the proper diagnosis of Vesicular Stomatitis, as infection is clinically indistinguishable from other vesicular diseases, such as foot-and-mouth disease, that infect livestock.<sup>1,3,6</sup> Differential diagnoses include foot-and-mouth disease, foot rot, and chemical or thermal burns. Vesicle fluid, epithelium from vesicles, or swabs from ruptured vesicles are the standard samples used in laboratory testing, though serum can be used as well; samples are then tested through various methods to detect the presence of virus or viral antigens.<sup>1</sup> Because insects appear to be important vectors in transmission of VSV, insect and pest control could serve as a chief method of preventing disease proliferation.<sup>1,3,6</sup> Caution should be exercised when dealing with outbreaks, and isolating infected animals could help prevent further spread within a herd.<sup>1</sup> Though potential vaccines against VSV have been explored, none have yet been discovered to be completely safe and effective for use.

The purpose of this study was to examine the localization and distribution patterns of viral replication in the epidermal layers of experimentally-infected cattle, using VSNJV-specific antibodies as part of an immunohistochemical protocol. Next, the distribution of adhesion

molecules were evaluated and characterized according to the anatomic location of the skin, such as thin haired skin where VSNJV lesions do not develop versus thick unhaired skin and mucosal surfaces where VSNJV lesions do develop, to determine any possible correlations between the distribution of adhesion molecules and VSNJV sites of replication.-

## CHAPTER 2: MATERIALS AND METHODS

#### Tissues

For this study, archived formalin-fixed paraffin-embedded blocks containing tissue from animals experimentally infected with vesicular stomatis were used. Animals were infected either via scarification or fly bite at the coronary band with vesicular stomatitis virus New Jersey serotype. Tissues were subjected to a series of examinations, including routine histopathology to assess morphologic changes, *in situ* hybridization for presence of replicating virus, and cell marker studies to determine types of inflammatory cells infiltrating into lesions. Those results are reported elsewhere. For the study reported here, these same tissues were used for immunohistochemistry to determine the distribution patterns of viral protein as well as types of cells infected.

#### Animals

Eleven 150-250kg Holstein steers were used in the previous experiment from which the tissues were obtained. All animal studies were conducted at the high containment USDA facility at Plum Island. For sake of completeness and understanding of the present study, the protocol for infection in that study is described here. Scarification (SC) inoculation was done on six animals, five scarified with virus and one with cell culture media only. Five animals were inoculated by fly bite (FB) – four with infected flies and one with noninfected flies. All animals

had hair from the area of inoculation removed with clippers followed by shaving 24 hours prior to the inoculation time. They were sedated with an intramuscular injection of Xylazine (1ml) prior to the inoculation. Post-inoculation, sedation was reversed by intravenous injection of 5ml Tolazuline.

Cattle were euthanized at 12, 24, 48, 72, 96, or 120 hours post-infection (HPI). Immediately after euthanasia, selected tissues (sections of the coronary bands, neck, flank, lip, and tongue) were collected into 10% neutral buffered formalin, allowed to fix for 72 hours, then processed to paraffin. Only the formalin-fixed material was transferred to UGA for analysis.

#### Virus

Virus utilized was a VSNJV field strain (95COB) isolated from a cow during an outbreak in Colorado, USA, in 1995.

#### Immunohistochemistry

Immunohistochemistry (IHC) was used to examine all tissue samples. Four primary antibodies were used. A polyclonal mouse anti-VSNJV (antibody to the VSV glycoprotein, kindly provided by Dr. Robert Tesh, University of Texas Medical Branch, Galveston, TX), was used to detect VSNJV in infected tissues. The monoclonal antibody p63, made in mouse, is a transcription factor specific for the nuclei of cells in the basal layer of the epidermis. The other two, polyclonal  $\alpha$ -catenin made in rabbit, and monoclonal Dsg 1 made in mouse, bind to adhesion molecules in the intercellular bridge structures between cells in the epidermis. The pertinent data for each of these primary antibodies are presented in Table 1.

Procedure for immunohistochemistry was as follows. Three to four micron sections were cut onto charged slides. Tissue sections were first deparaffinized in CitriSolv, and then allowed to air-dry until chalky white. Antigenic sites were exposed by incubating slides in a humid chamber with 0.1% trypsin solution in 0.1 M Tris pH 7.5 with 1.0% CaCl<sub>2</sub> at 37°C for 30 minutes. The trypsin was then inactivated by washing the tissues in 0.2 M Tris pH 7.5 with 0.1 M glycine twice for five minutes. Ultra V Block (LabVision) was used as a blocking solution against background staining, and the tissues were incubated at room temperature for 7 minutes, and then drained to remove excess solution. The slides were then washed for 5 minutes in DAKO Buffer (DAKO Laboratories), which was used a buffer solution in this protocol, before the primary antibody was applied.

Antibody	Dilution	Target in Tissue	Antibody Type	Species Used	Company/Provider
Anti-VSNJV	1:5000	VSNJV glycoprotein	Polyclonal	Mouse	Dr. Robert Tesh, UT Medical Branch
Anti-p63	1:100	Transcription factor in basal cells	Monoclonal	Mouse	Santa Cruz Biotechnology
Anti α-catenin	1:75	Intercellular adhesion molecules	Polyclonal	Rabbit	Santa Cruz Biotechnology
Anti- Desmoglein 1	1:100	Intercellular adhesion molecules	Monoclonal	Mouse	AbD Serotec

Table 1. Antibodies used in immunohistochemical studies in this experiment, including dilution used, whether monoclonal or polyclonal, and origin.

Once the primary antibody was applied at the standardized concentration (1:5000 for anti-VSV Ab, 1:100 for p63, 1:75 for α-catenin, and 1:100 for Dsg 1), the slides were incubated either for one hour at 37°C or overnight at 4°C; it was ultimately determined that incubating tissues with antibody overnight yielded better contrast under the microscope once the tissues were counterstained. DAKO Buffer was again used to wash the slides twice for 5 minutes at room temperature before and after the primary Antibody Enhancer (LabVision) was applied and incubated with tissues for 20 minutes at room temperature. A labeled Alkaline-Phosphatase Polymer (LabVision) was applied to the tissues, which were then incubated in a dark chamber at room temperature for 30 minutes, before a final wash in DAKO buffer two times for 5 minutes.

Vector Red (Vector Laboratories, Burlingame, CA) was used as the chromagen according to the following recipe per two slides: 2.5 mL 0.1 M Tris-HCl pH 8.2 solution + one drop Reagent 1 + one drop Reagent 2 + one drop Reagent 3. Tissues were incubated in a dark chamber at room temperature for approximately 12 to 30 minutes, though developing time was variable according to the tissue and antibody types being tested. Slides were rinsed in water before being counterstained with Mayer's Hematoxylin and mounted using permanent mounting medium (Permount).

#### Viral Antigen Distribution

The degree of intensity and distribution of virus, and its corresponding correlation with the investigated epidermal layers and intercellular structures, were evaluated by differential comparison of IHC staining results. Tissues were analyzed using a light microscope at the highdry setting (400X). Signal patterns and intensities were recorded according to the following

scores: no signal present (-); little to no positive signal with faint contrast (0/+); scattered to localized positive signal (+); generally well-distributed signal with good contrast to background (++); and extensive signal with very stark contrast and intensity (+++). Only the results from tissue sections with little to no background (non-specific) signal were considered in the final compilation of data, as the presence of significant background staining indicated errors in the IHC protocol or experimental technique, and also interfered with accurate discernment of true versus false positive signal. Approximately 20 high power fields were viewed per slide.

## CHAPTER 3: RESULTS

#### Presence of viral protein as determined by immunohistochemistry:

Results for the presence of viral protein are presented in Table 2. The signal associated with the presence of viral protein was always cytoplasmic and appeared as granular early in infection, and more diffuse as infection progressed. At no time was there any signal detected in tissues of the negative control animal. In the coronary bands harvested from the animal infected 12 hours previously, there was a light signal with small focus (Fig. 1). Then at 24 hours, a more widespread signal was observed (Fig. 2). Maximal intensity and distribution was seen at 48 hours (Fig. 3). At 72 hours, signal was still widespread, but intensity decreased towards 96 hours post-infection (Figs 4 and 5). At 120 hours, signal was faint and more punctate (Fig. 6). Viral antigen appeared to be confined to the mid-layers of the epidermis of the coronary bands, and its distribution was associated with vesicular formation, ulceration, and necrosis of tissue.

Animal Number	Tissue Source	VSV (1:5000)
12 HPI SC	RFCB <sup>a</sup>	(+++) <sup>b</sup>
	RRCB	(+++)
24 HPI SC	RFCB	(+++)
	RRCB	(+++)
24 HPI FB	RFCB	(+++)
	RRCB	(+++)
48 HPI SC	RFCB	(++)
	RRCB	(++)
48 HPI FB	RFCB	(+++)
	RRCB	(+++)
72 HPI FB	RFCB	(-)
	RRCB	(+++)
96 HPI SC	RFCB	(++)
	RRCB	(++)
96 HPI FB	RFCB	(+/++)
	RRCB	(+++)
120 HPI SC	RFCB	(+)
	RRCB	(+)

Table 2. Extent of VSV protein immunohistochemical signal seen in coronary bands

<sup>a</sup> RFCB indicates Right Front Coronary Band, RRCB indicates Right Rear Coronary Band; HPI – "Hours Post Infection"; SC – Scarification; FB – Fly Bite

<sup>b</sup> Scores were based on the following scale: absent signal (-); little to no signal (0/+); mild signal (+); moderate signal (++); strong signal (+++)

#### Anti p63 antibody

Results on immunohistochemistry for p63 are presented in Table 3. The p63 antibody displayed a consistently nuclear signal in the basal cells of the coronary bands, as well as in the flank skin with great intensity and contrast, but exhibited a more scattered distribution in the mouth tissues (lip and tongue, with the dorsal side more positive that the ventral side); neck skin was only faintly positive (Fig 7).

Animal	<b>Tissue Source</b>	p63 (1:100)
	1A (RFCB <sup>a</sup> )	$(+++)^{b}$
	5 (Neck)	(0/+)
Animal 1	22 (Tongue)	(++)
	24 (Lip)	(++/+++)
	25 (Flank)	(+++)
Animal 4	1A (RFCB)	(+++)
	1B (RFCB)	(+++)
	3A (RRCB)	(+++)
Animal 5	3C (RRCB)	(+++)
	3D (RRCB)	(+++)
Animal 7		
	1C (RFCB)	(+++)
Animal 8	1F (RFCB)	(+++)
	3E (RRCB)	(+++)
Animal 9	1A (RFCB)	(+++)
	1D (RFCB)	(+++)

 Table 3. Extent of immunohistochemical staining to delineate presence of p63 in cattle

<sup>a</sup> RFCB indicates Right Front Coronary Band, RRCB indicates Right Rear Coronary Band; HPI – "Hours Post Infection"; SC – Scarification; FB – Fly Bite

<sup>b</sup> Scores were based on the following scale: absent signal (-); little to no signal (0/+); mild signal (+); moderate signal (++); strong signal (+++)

#### *Anti* α*-catenin and Anti Desmoglein-1 Antibodies*

Results are presented in Table 4. Strong intercellular signal was consistently observed with  $\alpha$ -catenin in the strata spinosum and granulosum (mid-layers of the epidermis) in the lip, tongue, and coronary bands (Fig 8). The tongue in particular showed greater signal intensity on the dorsal (top) side of the tissue than the ventral side, and there was no signal in the hairy tissue just proximal to the the coronary bands. Very faint intercellular signal was found in the stratum spinosum of the flank tissue, and no signal in the neck skin.

Desmoglein 1 yielded comparable results with  $\alpha$ -catenin, with distinct intercellular signal in the deep layers of the stratum spinosum and the region where the transition between lesions and the stratum spinosum occurs (Fig 9); no signal was present where vesicles were forming or where the tissue was disrupted. Punctate signal was also found in the papillae of the tongue, and in isolated sebaceous glands of the coronary bands.

Animal	Tissue Source	α-catenin (1:75)	Desmoglein 1 (1:100)
	Coronary Band	(++) stratum spinosum	(-)
	Neck	(+) stratum spinosum	(-)
ANIMAL 1	Tongue	(++/+++) strata spinosum & granulosum	(-)
	Lip	(++/+++) strata spinosum & granulosum	(-)
	Flank	(0/+) stratum spinosum	(-)
	Coronary Band	(+++) stratum spinosum	(+/++) staining in sebaceous gland & epidermis
	Neck	(-)	(-)
ANIMAL 4	Tongue	(+) strata spinosum & granulosum	(0/+) stratum spinosum
	Lip	(+++) staining more intense in the upper stratum granulosum	(+/++) strata spinosum & granulosum
	Flank	(+) stratum spinosum	(-)
	Coronary Band	(+++) diffuse staining across whole strata spinosum/granulosum	(+) stratum spinosum
	Neck	(-)	(-)
ANIMAL 9	Tongue	(++) staining across whole strata spinosum & granulosum	(+/++) staining in papillae (projections)
	Lip	(+++) diffuse staining across whole strata spinosum/granulosum	(0/+) stratum spinosum
	Flank	(+/++) stratum spinosum	(-)

Table 4. Immunohistochemical signals for  $\alpha$ -catenin and desmoglein-1



Figure 1. Coronary band, VSV-positive steer, 12HPI, immunohistochemical signal reveals focal areas of viral protein, corresponding to site of scarification



Figure 2. Coronary band, VSV-positive steer, 24HPI, immunohistochemical signal reveals more extensive areas of viral protein, especially in the stratum spinosum



Figure 3. Coronary band, VSV-positive steer, 48HPI, immunohistochemical signal for viral protein reveals subtotal infection of the epidermis, with early vesicle formation



Figure 4. Coronary band, VSV-positive steer, 72HPI, immunohistochemical signal reveals very extensive infection of the epidermis, some vesicle formation, and marked edema of the underlying dermis



Figure 5. Coronary band, VSV-positive steer, 96HPI, immunohistochemical signal for viral protein is decreasing in intensity



Figure 6. Coronary band, VSV-positive steer, 120HPI, immunohistochemical signal for viral protein is restricted to small focal areas only



Figure 7. Coronary band, VSV-positive steer, immunohistochemistry for p63 highlights nuclei as round blue circles, only in the basal cell layer



Figure 8. Coronary band, control steer, <u>immunohistochemistry for  $\alpha$ -catenin</u> highlights the basal cell layer



Figure 9. Coronary band, control steer, immunohistochemistry for desmoglein highlights the intercellular bridges prominently within the stratum spinosum.

# CHAPTER 4: DISCUSSION

This study examined both the temporal and physical distributions of the Vesicular Stomatitis New Jersey Virus subsequent to infection in cattle. VSNJV replication was found to occur primarily in the keratinocytes of the stratum spinosum of the coronary bands. Differential positive signal was observed over time as infection progressed in the coronary bands, the site of inoculation for this study, where virus was detected. Viral replication was found to increase between 24 and 72 hours post-infection (HPI), with amounts of virus peaking at 48 HPI. The IHC signal was observed to have the greatest intensity and the most widespread distribution during these intervals, and this increase in signal corresponded with increasing vesicle formation.

The IHC signal for viral protein became more localized as the infection progressed to 96 and 120 HPI, with replication confined mainly to the viable peripheral cells immediately surrounding the vesicular lesions. One of the reasons for decreasing viral signal may be that the many of the cells initially infected became necrotic, falling out to result in the formation of clefts and vesicles. With fewer viable cells, there was less possibility for viral replication. The intensity and distribution of VSV signal at the site of inoculation was also consistent with the expected evolution of the host's natural defenses against VSV infection, which includes a strong innate immune response. Vesicular stomatitis is a known inducer of large amounts of interferon, which will rapidly dampen viral replication in susceptible cells<sup>3</sup>. Signal intensity then tapered off towards the end of the infection not only because of the increasing formation of lesions and

dead tissue (which was ultimately sloughed), but also because of the effectiveness of the immune system to defend against further infection to limit further spread.

In the past, both naturally-occurring and experimentally-induced infections, such as this experiment, of VSNJV in cattle have produced lesions in the thick skin of the coronary bands, mouth, gums, tongue, teats, and nostrils, with practically no vesicle formation in similarly-inoculated neck or flank skin.<sup>1,2,3,6</sup> In an earlier examination of the distribution patterns of VSNJV in cattle, Scherer (2006) proposed that the thinner layers of epidermis, such as those found in the flank skin where little to no viral replication was seen, lack the large keratinocytes necessary for proper viral proliferation and vesicle formation.<sup>2</sup> Thus it was histologic and structural differences between epithelial tissue that could explain why strongest signal for VSV replication was observed in the coronary bands (where virus was inoculated), mouth, nostrils, etc. rather than other areas of the body.<sup>2,7</sup>

In terms of physical distribution of VSV within the epidermis of specific infected tissues, replication was found to occur principally in the middle layers, namely the stratum spinosum and the stratum granulosum. The stratum granulosum and the stratum spinosum are distinguished from the other epidermal layers particularly, though not uniquely, by the presence of the intercellular bridge structures, such as desmosomes, which join adjacent cells tightly together.<sup>4,5,7</sup> These structures are composed of numerous adhesion molecules that are necessary for the epithelial tissue of the skin to maintain its sturdiness, as well as assist in its protective nature as a barrier to the environment; as a result, these intercellular bridges are prevalent in the thick epidermal tissues of the coronary bands, lip, and tongue where the levels of mechanical stress are high.<sup>2,4,5,7</sup> Though epithelial cells have a high rate of turnover, excessive cell destruction and tissue loss is prevented by the actions of these intercellular bridges and spiny processes.<sup>7</sup>

These results correspond with those observed by Scherer (2007), where viral antigens were detected in the upper layers of the skin, namely the strata spinosum and granulosum. Additionally, in this study it was found that cells where positive VSV signal occurred were also positive for cytokeratin, indicating the possible preference for keratinocytes for replication by the virus.<sup>2</sup>

The distribution of the virus within the different epidermal layers, such as the stratum spinosum and granulosum, was distinguished from the stratum basale using an anti-VSNJV antibody and a p63 antibody. Positive signal from the VSNJV antibody indicated presence of the virus because viral protein was detected, and positive signal from the p63 antibody, which stains specifically for a transcription factor in the nuclei of basal cells, identified the basal layer. Because p63 directly demarcated the stratum basale, and no signal for VSV was observed where signal for p63 occurred, it can be concluded that VSV replication likely does not occur in the undifferentiated basal cell layer of the epidermis.

Two antibodies, anti  $\alpha$ -catenin and anti Desmoglein 1, were used in this study to observe any correlation between viral distribution during replication and the presence of intercellular structures in the cells of the epidermis. Both antibodies bound specifically to adhesion molecules that compose the desmosomes, and positive signal was observed as a fine network around the edges of cells (where the microscopic bridges would be located). Desmoglein 1 and  $\alpha$ -catenin staining was found to occur chiefly in the stratum spinosum as well as the stratum granulosum. This pattern was comparable to that seen in an earlier study by Miragliotta of desmogleins and their distribution in dogs and cat epidermis (in relation to pemphigus complex), in which focal signal for desmoglein was found primarily surrounding the plasma membranes of keratinocytes in the suprabasal layers of the epidermis, specifically the stratum spinosum.<sup>4</sup> Miragliotta found

that differential positive signal was also observed to occur in the thicker layers of skin, such as the muzzle or footpads (as well as the muzzle of cattle), which had strong intensity and distribution of signal, versus the thinner layers, such as the abdomen and dorsum, that exhibited only light signal. The stratum basale exhibited no positive signal for the desmogleins.<sup>4</sup>

The positive signal generated by the desmoglein 1 and  $\alpha$ -catenin antibodies had the strongest intensity and distribution in strata spinosum and granulosum, the same two layers of the epidermis in which VSNJV replication was found to occur most prevalently, representing a very strong correlation between cell type and viral infection. Correlation does not equate with causation, however. This apparent preference at best reveals a possible connection between cells that contain prominent intercellular bridges between them and viral replication, but no definite conclusions can yet be drawn. These observations may, however, provide grounds for future explorations into intercellular structures, especially because the cell receptor for VSNJV is unknown. Much of the VSNJV's pathogenesis is still only partially understood, and more research is needed to shed light further on the subject.

The first conclusion of this study is that VSNJV replicates in keratinocytes of the stratum spinosum and granulosum of the bovine coronary bands. Second, the distribution of epidermal adhesion molecules coincides with the layers of the epidermis where VVNJV replicates and causes vesicles. Finally, a marked lower expression of adhesion molecules is present in thin haired skin sites where the virus does not replicate. This study demonstrates for the first time association between expression of epidermal adhesions molecules and VSNJV lesion development

## WORKS CITED

- 1. The Center for Food Security and Public Health, College of Veteriary Medicine, Iowa State University, Ames, Iowa, 50011. Institute for International Cooperation in Animal Biologies. 2006 Jan. Available at: http://www.cfsph.iastate.edu. Accessed Jan-May 2010.
- Scherer C., O'Donnell V., Golde W., Gregg D., Estes D.M., Rodriguez L.L.. Vesicular Stomatitis New Jersey virus (VSNJV) infects keratinocytes and is restricted to lesion sites and local lymph nodes in the bovine, a natural host. Vet. Res. 38 (2007) 375-390. EDP Sciences, 2007. Available at: http://www.edpsciences.org/vetres.
- 3. Letchworth G.J., Rodriguez L.L., Barrera J., Review: Vesicular Stomatitis. The Veterinary Journal 1999, 239-260. Article No. tvjl.1998.0303. Available at: http://www.idealibrary.com.
- 4. Miragliotta V., Coli A., Ricciardi M., Podestà, Abramo F. Immunohistochemical analysis of the distribution of the desmoglein 1 and 2 in the skin of dogs and cats. AJVR, Vol. 66, No. 11, November 2005.
- Miragliotta V., Donadio E., Felicioli A., Podestà A., Ricciardo M.P., Ceccardi S., Abramo F. Immunolocalisation of desmoglein-1 in equie muzzle skin. Equine Veterinary Journal (2006) 38 (5) 485-487.
- 6. Reis, Jr. J., Mead D., Rodriguez L., Brown C. Transmission and pathogenesis of vesicular stomatitis viruses. Brazilian Journal of Veterinary Pathology, 2009, 2(1), 49-58.
- Dellman's Textbook of Veterinary Histology, 6<sup>th</sup> Edition. Eurell J., Frappier B.; Blackwell P. Blackwell Professional Publishing, 2121 Avenue, Ames, Iowa, 50014, USA, 2006.