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Presence and Persistence of Foot-and-Mouth Disease Virus in Bovine Skin

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ABSTRACT

GAILIUNAS, PETER (Plum Island Animal Disease Laboratory, Greenport, N. Y.), AND GEORGE E. COTTRAL. Presence and persistence of foot-and-mouth disease virus in bovine skin. J. Bacteriol. **91**:2333–2338. 1966.—This study established that the seven known antigenic types of foot-and-mouth disease virus (FMDV) have consistent affinity to all areas of bovine skin, even though gross cutaneous lesions usually are found only in the pedal area. Considerable amounts of FMDV were present in skin of 13 different body areas, irrespective of the presence of hair. All skin specimens from the trunk of 50 experimentally infected steers, necropsied from 12 hr to 7 days postinoculation (DPI), contained FMDV in the dermal and epidermal tissues. In skins of some steers, FMDV persisted for as long as 5 days after cessation of viremia. The highest average virus titer, $10^{3.6}$ plaque-forming units (PFU) per g of skin, was found at 2 DPI. Some areas of the trunk and extremities had titers of approximately $10^{5.0}$ PFU per g of skin. Characteristic gross lesions were not observed in sampling areas. The present observations have epizootiological importance for hides offered in international trade, because FMDV localized intracutaneously is more difficult to inactivate than virus adhering to hide surfaces.

This study was conducted to obtain basic information on the occurrence of foot-and-mouth disease virus (FMDV) in bovine skin not affected by characteristic gross lesions. Although the hazards of disseminating FMDV through hides of cattle were recognized nearly 40 years ago (13), reports concerning occurrence of FMDV in various skin areas of natural hosts are not available. The Fourth Progress Report of the British Foot-and-Mouth Disease Research Committee (6) described the detection of FMDV in a salted hide stored at "outside temperature" for 46 days, but failed to show that infectivity of the specimens tested was not due to surface contamination. The consensus of published opinions seems to be that FMDV has no affinity for normal hairy skin (5, 12). The dissemination of FMDV through hides was attributed to contamination of their surfaces with viremic blood or other infectious materials (15). According to Belin (3), FMDV propagates in all deep epithelial layers which are in an active state of regeneration, and the vesicles develop wherever superficial layers of the skin do not obstruct their eruption, like interdigital spaces, mouth, and inner thigh of cattle with thin skin. Belin further stated that the remainder of the cutaneous surface is free of evidence of the development of visible lesions. Localization of the FMDV, after the viremic stage, was believed to be in the epithelial tissues (11). Platt (14) pointed out that the localization of FMDV lesions is strictly limited to the sites of predilection, and that their extension to the hairy skin of the legs rarely occurs.

This report presents observations on the occurrence, persistence, and distribution of FMDV throughout various areas of bovine skin.

MATERIALS AND METHODS

Viruses. Strains representing all seven known types of FMDV were used. Four were Argentine field strains, A-1, O-2, O-9, and C-3, and one was a European field strain from The Netherlands, O-Mulder. In addition, one Asian and three African strains were used, Asia-1 PAK-1, SAT-1, RV-11, SAT-2 RHO-1, and SAT-3 RV-7. All viruses were passed only in cattle, and were stored at −50 C. Further history of the virus strains, passage levels used, and storage conditions have been reported (4).

Cattle. Grade Hereford steers and heifers, 16 to 24 months old, weighing from 270 to 320 kg, were used as a source of skin samples and also for confirmation

of the infectivity of the virus present in the skins. Before delivery to the laboratory, the steers were raised under average North American range conditions. They apparently were healthy and had clean hair coats. Steers were held in laboratory isolation rooms from 2 days to 5 weeks, and were fed chopped alfalfa hay. The temperature in the rooms was approximately 21 C, and the relative humidity was 50%. As the study continued throughout a year, possible seasonal and climatic influences on the susceptibility of bovine skin to FMDV were dealt with at random.

Tissue cultures. The production of primary monolayer tissue cultures from bovine kidney cells in 4-oz (about 120 ml) prescription and 5-liter Povitsky bottles has been described (2).

The medium for cell growth was composed of Hanks balanced salt solution, containing 6% bovine serum, 0.5% lactalbumin hydrolysate, and 0.01% phenol red (LC medium). The same medium, but without serum, was used as maintenance fluid for virus growth in Povitsky-bottle cultures.

Source and collection of samples. Cattle, used as a source of skin specimens, were inoculated on the tongue, except five which were infected intramuscularly. To prepare the inoculum, the infected tongue epithelium or other tissues were weighed, ground with Alundum, and diluted 1:10 or 1:5 with Tryptose Phosphate Broth (Difco) or LC medium. After centrifugation at 880 × *g* for 20 min, the supernatant fluid was collected, and penicillin G sodium (10,000 units) and dihydrostreptomycin (10 mg) were added for each milliliter of suspension. The volume of inoculum by the intramuscular route was 5 ml, and on the tongue, 1 to 5 ml. The amount of virus in the inoculum varied from approximately 6–12 to 5 millions of bovine infectious doses per milliliter. Cattle showed clinical signs of foot-and-mouth disease at 24 hr when inoculated on the tongue, and 2 or 3 days postinoculation (DPI) when the intramuscular route was used. They were slaughtered at selected intervals during the viremic and convalescent stages of the disease. Some inoculated cattle had normal temperatures and manifested no clinical signs of infection when slaughtered.

The cattle were taken singly to an autopsy room (which was decontaminated with 2% sodium hydroxide solution), stunned with a captive bolt pistol, and decapitated. In selected cases, heparin-treated blood was taken for viremia titrations. The carcasses were suspended with an electric hoist, and an individual who was not previously in contact with the animals collected hide samples from areas not visibly contaminated with blood. Precautions were taken to avoid transfer of virus to specimens from other tissues or body fluids.

Generally, from one to four skin samples were taken. The sampling areas were selected arbitrarily to represent thick skin with dense hair (lumbar); thin skin with dense hair (shoulder); thick skin with sparse hair (perineal); and thin skin with smooth hair (inner thigh). The lumbar specimens were from the area above the lumbar vertebrae, approximately 5 cm lateral to the vertebral column. The shoulder specimens were from the area covering the deltoid muscle. The perineal specimens were from the area approximately 5 cm inferior to the anus. The inner thigh specimens were from the midway area between the symphysis pelvis and the hock joint. To determine the regional distribution of FMDV in skin, additional samples were taken from areas covering the cheek, lateral neck, ventral dewlap, ventral brisket, ventral udder, ventral abdomen, Achilles insertion, anterior carpus, and lateral aspect of the first phalanx, about 2 cm above the coronary band.

Before collecting the samples, the skin was shaved by wetting the hair with cold tap water. Strips of skin, approximately 5 by 8 cm, were carefully removed to be as free as possible of subcutaneous tissue, and were immediately rinsed in tap water under maximal faucet pressure. The water temperature was about 20 C, with a *p*H of about 6.5. Subsequently, each sample was placed in a sterile screw-capped bottle and transferred from the autopsy room to the laboratory, where it was washed, again successively, in three beakers, each containing approximately 200 ml of LC medium. After the last washing, the samples were placed in sterile specimen bottles and stored at −20 C until virus assays were made.

Preparation of inoculum. Thawed hide samples, weighing approximately 10 to 25 g, were cut with scissors into irregular pieces of approximately 2 by 2 by 5 mm. They were triturated with Alundum and diluted 1:3 or 1:5 by weight with LC medium. Because of the elasticity of the skin, only a part of the tissue could be ground into a suspension. After centrifugation at 880 × *g* for 20 min, approximately 30 to 90 ml of the supernatant fluid was collected and either tested immediately or frozen at −20 C for later assay.

For an indication of the relative amounts of FMDV in dermis and epidermis, samples of shaved and washed lumbar skin were divided into narrow strips and then cut, apportioning an estimated one-fourth of the total skin thickness to the epidermal part, and the balance to the dermal part. Thus, the epidermal part included some of the subjacent connective tissue. In a few instances, the epidermal layers, as determined by pigmentation, were scraped off with a sharp knife and assayed for virus in comparison with whole-skin virus content.

Virus assay. Quantitative and qualitative assays for FMDV in hide samples and blood were made in tissue cultures by both the plaque (1) and Povitsky-bottle techniques (7) and by inoculation of steers. For titration, 0.1 ml of each 10-fold serial dilution was inoculated into three prescription-bottle cultures, and titers were calculated per gram of skin sample or milliliter of blood.

When it was found that the virus concentration was very low, 1-ml amounts of 1:3 or 1:5 suspensions were assayed by the plaque technique, with 10 cultures. If virus was not detected in the 1-ml suspension, Povitsky-bottle cultures, containing 1,000 ml of LC medium, without serum, were inoculated with skin suspensions ranging from 30 to 90 ml. For comparative titer, a few samples were titrated in steers and by the plaque technique. To confirm infectivity for the natural host, skin suspensions (2 to 5 ml), usually from the lumbar area, were inoculated into tongue epithelium of steers.

pH measurements. The *p*H of epidermal and subcutaneous surfaces of representative skin samples from steers infected with FMDV was determined with a zeromatic Beckman *p*H meter.

TABLE 1. *Occurrence of FMDV in skin specimens from the trunks of steers killed during viremia and convalescence**

Steers killed (DPI)	No. of steers examined	Virus assay of skin specimens			Average virus titer (log_{10} PFU/g)
		Tissue cultures		By steer inoculation	
		Prescription bottle	Povitsky bottle		
1	10	10/10	—	6/6	3.2
2	12	12/12	—	4/4	3.6
3	7	7/7	—	2/2	3.2
4	8	8/8	—	5/5	2.9
5	5	5/5	—	2/2	2.5
6	4	4/4	—	1/1	2.1
7	4	4/4	—	2/2	1.1
8	8	4/8	5/8	2/2	1.0
9	3	0/3	3/3	2/2	—
10–15	5	0/5	0/5	—	—
16–34	7	0/7	0/7	—	—

* Numerator = number of whole skins positive; denominator = number of skins tested; — = not done; DPI = days postinoculation.

RESULTS

Occurrence and persistence of FMDV in skin. Examination of the specimens taken at necropsy demonstrated the presence of FMDV in the shaved skin from the trunk of each of 50 experimentally infected steers killed between 1 and 7 DPI. Of 11 steers killed 8 and 9 DPI, virus was found in the skins of 8. The blood of infected steers used in the present study contained FMDV no longer than 4 DPI. Thus, FMDV was present in the skins of most cattle 4 or 5 days after cessation of viremia. Steers killed between 10 and 34 DPI had no demonstrable virus in the skin (Table 1).

Considerable amounts of virus were present in the skins of steers necropsied 12 and 16 hr after tongue inoculation (Table 2, steer no. 1 and 2). Two steers, inoculated with several bovine infectious doses of FMDV, appeared healthy, clinically, and had no gross lesions at necropsy. Their skin specimens, however, yielded infectious titers of $10^{2.6}$ and $10^{1.8}$ plaque-forming units (PFU) per g of lumbar skin, respectively.

All seven principal antigenic types of FMDV tested were found to have an affinity for skin (Table 2). The infectivity of FMDV isolated

TABLE 2. *Presence and persistence of seven types of FMDV in four representative areas of bovine skin**

Steer no.	Killed (PI)	Virus strain	Virus titer in blood and skin samples				
			Blood	Lumbar	Shoulder	Thigh	Perineal
1	12 h	O-9	5.1	2.6	—	—	—
2	16 h	O-9	5.5	1.8	—	—	—
3	1 d	O-9	5.5	—	—	—	5.5
4	1 d	O-2	5.2	3.6	4.5	3.6	4.5
5	1 d	Asia-1	5.3	3.8	—	—	—
6	2 d	SAT-2	—	3.4	3.8	3.5	3.0
7	2 d	A-1	4.5	3.2	—	—	—
8	2 d	SAT-3	4.1	2.0	2.0	2.0	1.5
9	2 d	C-3	4.0	4.7	4.6	4.1	—
10	3 d	SAT-1	3.6	2.5	3.2	3.1	4.3
11	3 d	O-Mulder	—	3.0	4.0	3.2	2.7
12	4 d	C-3	2.7	2.5	4.0	3.8	3.0
13	4 d	O-2	2.0	2.0	2.8	2.5	3.0
14	5 d	O-9	N	4.2	—	—	—
15	5 d	O-2	N	2.7	2.0	1.8	1.5
16	5 d	SAT-1	N	2.0	—	—	—
17	6 d	O-2	N	1.0	2.1	1.3	N
18	6 d	O-9	N	2.6	—	—	—
19	7 d	A-1	N	1.0	—	—	—
20	7 d	O-2	N	1.5	1.0	1.0	—
21	8 d	C-3	N	—	—	1.3	—
22	8 d	O-9	N	P	P	—	—
23	9 d	O-9	N	P	P	N	N
24	9 d	O-9	N	P	P	N	N
25	10 d	SAT-2	N	N	N	—	—

* Log_{10}PFU per ml or g; P = positive, trace of virus detected by Povitsky bottle tissue culture assay; N = negative; — = not done; PI = post-inoculation; h = hours; d = days.

from skins was confirmed by the inoculation of 24 steers, each receiving a sample suspension from a different animal's skin. All recipient steers developed clinical signs of FMDV between 1 and 4 DPI. The sample-collection procedure was designed to prevent any accidental transfer of virus to the samples from other tissues or from the environment, during postmortem. The reliability of this procedure was tested by comparative titrations of duplicate samples from the same skin area. One sample was washed by the technique described, and was assayed for virus immediately. The other sample was held submerged for 24 hr in 1:10,000 sodium bifluoride solution at *p*H 3.8, and was assayed after neutralization of the surface acidity. Both samples yielded comparable titers. Thus, the FMDV in skin samples has localized intracutaneously, in vivo, probably by hematogenous dissemination. Detection of FMDV in the lumbar and other skin samples 12 hr after tongue inoculation makes the entry of virus into the skin from an extraneous source highly unlikely.

Amount and distribution of FMDV in skin. Relative amounts of FMDV found in different representative areas of skin at 1-day intervals after infection are given in Tables 2 and 3. The average titers of virus in the skin of the trunk, determined by examination of a specified number of steers killed during viremia and convalescence, are given in Table 1. The highest concentrations of virus, approximately $10^{5.0}$ PFU per g, were found in the skin of extremities (Achilles insertion and anterior carpus; Table 3). The highest titers of virus in the skin of the trunk were in the perineal, lumbar, and shoulder areas (Table 2). In some skin samples, the amounts of virus approached but never exceeded the amount of virus contained in the blood at the peak of viremia. Testing of four representative areas of skin (Table 2) demonstrated that there was no apparent relationship among the thickness of the skin, hair density, and concentration of virus in a given body area. The udder, abdomen, and inner thigh are areas which, reportedly, sometimes develop characteristic skin lesions. However, according to present findings, these areas had the same or lower concentrations of FMDV than those parts of the body not known to develop lesions.

Limited assays indicated that most of the FMDV present in the skin localizes in the epidermal and, perhaps, the most superficial layers of the dermis (Table 4). Surface scrapings of the shaved skin had titers approximately 1 log unit higher than the whole skin. It is improbable that these scrapings contained any components of the dermis. Simultaneous titrations of skin samples in cattle and tissue cultures gave comparable values within the limits of normal variations, e.g., an Achilles-insertion-area sample yielded an infectious titer of $10^{5.7}$ bovine ID_{50} and $10^{5.2}$ PFU in tissue culture per g of skin.

The effect of mechanical traumatization of skin also was investigated. The skin of the lumbar region of a steer was shaved and part of the area was superficially scarified, preceding inoculation on the tongue. When the steer was killed 2 DPI, the titer of FMDV in PFU per g was $10^{7.3}$ in scrapings of scarified skin, $10^{6.0}$ in superficial layers from a symmetrically located nonscarified skin area, and $10^{5.8}$ (per ml) in blood.

TABLE 3. *Regional distribution of FMDV in bovine skin during viremia and early convalescense*

Samples	Virus titer*					
	Heifer 1 (1 DPI)	Steer 1 (2 DPI)	Steer 2 (3 DPI)	Steer 3 (4 DP)I	Steer 4 (6 DPI)	Heifer 2 (7 DPI)
Blood	5.2	4.7	3.6	2.0	N	N
Cheek	3.5	3.8	3.5	1.2	N	N
Lateral neck	—	2.5	1.8	—	1.7	—
Ventral dewlap	4.0	3.2	4.6	—	N	—
Shoulder	4.5	2.5	3.2	2.8	2.1	0.7
Ventral brisket	3.4	3.5	—	3.3	1.7	—
Dorsal lumbar	3.6	4.0	2.5	2.0	1.0	0.7
Perineum	4.5	3.2	4.3	3.0	N	N
Ventral udder	3.8	—	—	—	—	N
Ventral abdomen	—	3.6	2.8	3.6	1.0	—
Inner thigh	3.6	3.3	3.1	2.5	1.3	N
Achilles insertion	4.4	3.7	—	5.2	3.0	1.2
Anterior carpus	—	4.9	4.0	—	2.8	N
First phalanx	—	3.4	5.8	—	3.3	2.0

* Log_{10} PFU per ml or g; N = negative; — = not done; DPI = days postinoculation cattle killed.

TABLE 4. *Comparative amounts of infectious FMDV in bovine blood, whole lumbar skin, superficial skin layers, and dermis during viremic and convalescent stages of infection*

Cattle no.	Killed (DPI)	Virus titer*			
		Blood	Whole skin	Superficial layers	Dermis
1	1	5.2	3.6	4.1	2.7
2	2	4.7	4.0	4.6	3.4
3	3	3.6	2.5	3.7	1.5
4	4	2.0	2.0	4.0	1.7
5	5	N	2.7	4.0	1.5
6	6	N	1.0	2.2	1.3
7	8	N	1.7	1.2	N

* Log_{10} PFU per ml or g; N = negative.

Comparable results were obtained in a replicate test. Thus, mechanical traumatization seemed to increase the amount of virus in skin.

Other observations. Generally, characteristic gross lesions were not observed in the sampling areas of the skin, regardless of whether the steers were necropsied while apparently healthy or when they had fully developed lesions at the sites of predilection. In some steers, Achilles-insertion areas showed patches of superficial fresh or crusted epidermal erosions, presumably caused by some mechanical injury. The average *p*H of the infected skin specimens was 7.6 on the epidermal surface and 7.8 on the subcutaneous side.

DISCUSSION

These observations provided evidence that FMDV has a consistent and, generally, grossly inapparent affinity for all the areas of bovine skin. The distribution of significant concentrations of FMDV in all the cutaneous parts of the body during the usual course of the disease is a fixed characteristic of all seven known types of FMDV. The persistence of virus in the skin after viremia indicates that virus particles are able to passage vascular endothelium of the dermis and may be trapped by the pericytes or other local phagocytes which protect them against circulating antibodies. It is unlikely that a barrier, preventing the entrance of FMDV from the dermis to the epidermal layers, exists in the bovine skin. A true basement membrane was not apparent in the skin of Hereford and Aberdeen cattle (9).

Absence of grossly visible cutaneous lesions, except in the interdigital and coronary skin, does not exclude the possibility that FMDV may proliferate to some extent and may form microscopic lesions in hairy skin, with a preference for areas where the viral concentrations are high. Microscopic foci of viral-type acantholysis were reported in the teat skin of heifers infected with FMDV (8). Similar changes might occur in areas such as carpal and hock joints, which are exposed to constant mechanical insults accompanied by intense loss of cells and compensatory active regenerative processes in the germinative layer. It is possible that, in such areas, virus persists longer than was found in this study, and it could be shed in the desquamating cornified cells.

Epizootiologically, these observations are important for hides offered in international trade, because FMDV lodged intracutaneously is more difficult to inactivate than virus adhering to hide surfaces. The presence of FMDV in the skin at an early and inapparent stage of infection creates additional difficulties in preventing the dissemination of the virus through infected hides. Furthermore, as studies now in progress in this laboratory indicate, FMDV may survive in cattle hides held at lower temperatures for months and resist drying for weeks. In limited sampling, FMDV of high titer was found in areas of porcine and ovine skins not affected by gross lesions. It is probable that FMDV occurs in skins of all susceptible hosts. Huck (10) classified FMDV in a group of picornaviruses and a subgroup of enteroviruses. The facts revealed by these observations suggest the possibility that other picornaviruses may also possess an affinity for the epidermis and may persist in normal-appearing skin.

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