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TO IMPROVEMENT OF EVIDENCE BASE: THE FORENSICS OF THE PORCINE PSEUDORABIES CASE

Buzun A.I., Koltchik O.V., Bobrovitska I.A., Stegniy A.B., Vovk S.I.
National Scientific Centre "Institute of Experimental and Clinical Veterinary Medicine", Kharkiv, Ukraine, e-mail: epibuz@ukr.net

Yatsenko I.V.
Kharkiv State Zooveterinary Academy, Kharkiv, Ukraine

The outbreaks of porcine devastating infections in contexts of modern situation with African swine fever in the Russian Federation must necessarily be regarded primarily as presumably criminal act – to exclude and to block the malicious actions of real enemies on time. Unfortunately, there are absent of a good forensics practice for collection evidences of the agroterrorism or biological crimes (biocrimes) in Ukraine now. On case of pseudorabies outbreak in small farmer holding were developed the rules of sampling places and collection of specimens simultaneously for etiological and Para-criminal/causal reasoning investigations. Etiological reasoning was conducted by the standard operation procedure (SOP IECVM #12-2013) that is based on OIE Diagnostic Manual. Causal reasoning was conducted by experimental approaches that include rectal aerobic and anaerobic Bacillus spp. studies by its pools of spores and lytic phages trials. These pools were isolated from rectal swabs of separately target, suspicious and mock swine herds by general acceptance methods for spores and bacteriophages isolation. As criterions of causal reasoning the lytic activity of accordance phages pool for homologous (target swine herd) and heterologous pools (suspicious swine herd) of aerobic and/or anaerobic Bacillus spp. were counted. Pseudorabies virus and antibodys were estimated in brains samples of infected piglets and serums of its sow and pigs from target and suspicious herds respectively. Moreover, the phages pools from target swine herd have lytic activity on Bacillus spp. aerobic and anaerobic cultures that were isolated from target (farmer holding) and suspicious swine herds (homestead piggeries 3), but not in mock swine herd (homestead piggeries 1). Our experimental methodology is perspective to establishing of the origin etiological agent in range circumstances of epidemiological-causal investigation or in evidence collection for criminal proceedings. It also need further examination and possible improvement for the verification of methodology – particularly regarding the reproducibility and standardization

Keywords: Pseudorabies, swine heards, sampling, forensics, Bacillus spores, Bacillus bacteriophages.

In view of criminal law the nature of epizootic emergency situations (hereinafter – emergency cases) has a three main evidence-based origins: 1 – result of natural disaster (usually without judicial consequences); 2 – due to negligence or incompetence responsible persons/officials (biocrames) and/or 3 – as a result of agroterrorism [1]. The last two items are the kinds of presumably crimes that should be investigated with approaches of forensics: i.e. evidence-based of pre-judicial investigation. The sampling of specimens in full size and a good practice of handling with forensic microbial specimens have basic significance to objective justice. If specimens for forensics taken by wrong way we will not collect the objective evidence for court. To the same consequences will lead the contamination of samples during collection, handling, transport or storage as well as the degradation of samples during performing of these procedures [2]. The outbreaks of African swine fever (ASF) in Ukraine in contects of modern situation must obligatory be regarded primarily as presumably criminal act – to exclude and to block the malicious actions of real enemies on time. Unfortunately, there are absent of a good forensics practice for collection evidences of the agroterrorism or biologiocal crimes (biocrimes) in Ukraine now. This article is our first attempt to elevate issues related to the practices of sample collection and initial handling of field specimens for forensics of ASF during collection evidence of malicious actions against Ukrainian piggeries.

Material and methods. Selection of sampling places and collection of speciments – Present investigation was motivated by obligatoriness explanation to owner the Pseudorabies outbreak causes in small farmer holding (83 pigs in total, 2 boars, 8 main sows) at winter-2015 in Lugansk region (item 2 of table 1). Disease had clinical signs in piglet nest under nursing sow (item B_2 of table 1) which was inseminated 3.5 month ago with boar from homestead piggeries of owner friend (items 3 and A_3 of table 1). Another nest of this holding (item C_2 of table 1) was a progeny of local boar of this holding (items A_2 of table 1) and had clinical healthy status as their parents too. Sampling for evidences assembling was conducted in two patterns: of aetiological and causal reasoning. For aetiological reasoning brain specimens were taken from infected piglet and the blood of serums – from their parents (case 2, items A_2 and B_2 of table 1) and from pigs of suspicious homestead piggeries (case 3, items A_3 and B_3 of table 1). For the causal reasoning rectal swabs were taken from parents of infected piglets (from boar – item A_2 and sow – item B_2 of table 1), sow with healthy progeny at neighbor cage (item C_2 of table 1), two pigs at suspected homestead piggeries № 3 (items A_3 and B_3 of table 1) and from two pigs at control homestead piggeries (№ 1 of table 1) of the same village (items A_1 and B_1 of table 1) which hadn't links with mentioned farmer holding and suspicious homestead piggeries. The aetiological reasoning were conducted in frame of the standard operation procedures for pseudorabies diagnosis (SOP IECVM #12-2013), which are based on OIE Manual [3]. Briefly, SOP IECVM includes procedures for: 1 – neurotropic virus/bacteries isolation and identification and 2 – serological assays for retrospective analysis of field cases. So, the brain specimens

were investigated by virus isolation procedures "rabbit assay", then agent cultivation was done by consecutive passages on PK-15 cells (for the purification and enhancement of virus titre of more than 10^2 $TCID_{50}/cm^3$) and, finally, virus identification (in dose 10^2 $TCID_{50}$) – by the neutralization test using the reference serum from OIE Reference Laboratory (PIWet, Pulawy, Poland, dilution 1:25). The serological investigations screening test (indirect haemagglutination, IH) was applied using "ReproSuiScreen-IECVM" kit (NSC "IECVM", Kharkiv, Ukraine) and confirmatory test (ELISA) – using kit for IH-positive specimens (IDEXX, USA). The Causal reasoning were conducted by experimental approaches that include bacterial spores and phages pools isolation from the mentioned rectal swabs. These approaches were realized by modification of "Turkish technology" (SOPs of Kafkas University, t.Kars, Turkey) of B. anthracis soil spores and phages isolation [4]. Briefly, the rectal swab suspended with sterile distilled water (SDW) in 1:5 ratio (total volume usually 2.5 ml) and mixed well by shaking. Half of the volume (usually 1.0 ml) of each supernatant was treated by heat at 62.5–63.0 °C (water bath) during 15–20 minutes untill the general pool of bacterial spores will be isolated. For aerobic Bacillus spp. growthing, one part of termoinactivated sample was plated on agar plates (brain-heart infusion broth from Sigma-Aldrich Co. with 1.6 % Bacto agar Difco in final concentration) in duplicate volume of 100–150 µl. For Clostridium spp. growthing, second part of termoinactivated sample was plated on anaerobic agar plates (M228, HiMedia Laboratories Pvt. Lim.) using the same method. Plates were incubated at 37 °C overnight or for 48–72 hours at aerobic and anaerobic conditions, respectivelly. Plates were checked for Bacillus spp. using the Gram staining method. For the phage isolation from rectal swabs we used routine method of lytic phages isolation with a host and without the inducers. Briefly, the second part of each specimen extract on SDW (see above) was combined with the equal volume (usually 1 ml) of Brain Heart Infusion Broth (Sigma-Aldrich Co.). This sample was devided on two equal half (each at about 1 ml) after mixing and incubated at 37 °C for 2 hours. Followed this, 3 ml of a mid-log phase culture [2] of the gomologous (i.e. from the same pig) pools of the Bacillus spp. (as mentioned above – aerobic and anaerobic isolates) were added separately and all mixtures were incubated at 37 °C overnight in aerobic and anaerobic condition, respectively. At the next day, top 3 ml of culture was harvested and filtered using a 0.22 µm membrane filter (Syringe-filter, product #99722 of TPP Co.) to removing any bacteria and rough debris. The filtrate was stored at 4 °C until required. To determine the lytic activity of phage recovered using the method of the plaque assay performed on mentioned agar plates with aerobic and anaerobic daily cultures of the gomologous (i.e. from the same pig) pools of the Bacillus spp. that were used as a host. The plaque assays were carried out according to the method of Abshire T.G., 2005 [5]. Brief, the host of bacterial inoculum for phage propagation was prepared by transferring 5–6 isolated colonies from the agar-culture, that grown overnight to 5 ml at sterile 10 mM phosphate-buffered saline (PBS), pH 7.2. Agar plates were inoculated with 100 µl of the bacterial suspension of studied pools, was spreaded with a disposable plastic spreader until absorbed and 15 µl of the phage suspension was delivered on agar surface. After fluids absorption, the plates were incubated at 37 °C overnight in the inverted position. The cultures were inspected for plaque formation at regular intervals. To increase the concentration of enteric phages we mixed an equal volumes of phage filtrate with a mid-log phase culture of appropriate bacterial isolate from studied pools and incubated at 37 °C overnight. At the next day the culture was spin down (8000 × g for 10 min), and supernatant filtered through 0.20 µm membrane filter. The phage titer was determined by performing a plaque assay by general acceptance routine test dilution (RTD). This process repeated until a sufficiently high-titer phage stock obtained (to 10^5–10^7 bacterial litic activity units, BLU_{50}/cm^3). Determination of the enteric phages "host range". The turbidity of an overnight cultures of the different aerobic and anaerobic bacillus pools, which were isolated from corresponding samples (see above) in peptone broth was adjusted to a Mc Farland standard of 0.5 ($1.5x10^8$ cfu/ml) using isotonic peptone-saline. The surface of agar plate was covered by 0.2 ml of this suspension and lefted to dry at 37 °C for 20 minutes. 10 µl of each phage isolate with standard activity $10^5 BLU_{50}/cm^3$ was dropped onto correspont sector of the inoculated plate's surface and lefted to dry for 10 minutes. After that, the plate was incubated at 37 °C and examineted for the presence of plaques at 24-hour intervals. The result was considered as positive (correspondent bacterial pool sensitivity to studied phage) in case of the formation of spots with the different levels of transparency (see table 1) on the agar cultures surface of appropriate bacterials pool.

Results and discussion. The aetiological investigation has lead to isolation and identification of pseudorabies virus and the bacterial concurrents microflora:

- appearance of pruritic rabbit was registered at 3rd day after inoculation;
- cytopathic agent was isolated in cell culture (after sterilizing filtration lung suspension through 0.22 µm membrane) in the presence of the antibiotic during two blind passages and raised titre to level of 4 lg throughout 2 additional consecutives passages;
- 100 cytopathic units of this agent were fully blocked by pseudorabies reference serum but not by other reference serums from porcine neuroinfection kit of the IECVM – against porcine enterovirus encephalomyelitis (PEE), porcine viral encephalomyocarditis (PVE), listeriosis and streptococcosis);
- in addition to viral agent from serum specimens of holding № 2 (items A_2 & B_2 of table 2) and from homestead piggeries № 3 but not from № 1 (see table 1), the antibodies against pseudorabies virus in titres 1:4–1:64 were registered by the serum neutralization reaction (SN) with 100 units of reference virus (strain «18v-IECVM»). These samples had zero titers by SN with 100 units of against PEE- and PVE-viruses as well as in bt agglutination tests with listeriosis and streptococcosis (suis) whole-bacteria antigens;
- the apart viral agent pure culture of unidentificated Mycoplasma spp. was isolated from specimens of piglets brains, which were revealed in samples of piglet surums of holding № 2 but not in serum samples from pigs of homestead piggeries № 1 and 3 (see table 1).

The results of causal investigation were summarized in table 1. As we can see, each of all three different piggeries had specific profiles of enteric microbioma consorties "Bacillus spp. – Their phages". The sensitivity of Bacillus spp. pools to phage isolates ("host

range" of phages isolates) in cases of diseased pigs in piggery № 2 (items A_2 & B_2 of table 1) and pigs from suspected homestead piggeries № 3 (items A_3 & B_3 of table 1) but not pigs from "control/ mock" homestead piggeries № 1, that didn't have any links with piggeries № 2 and 3. Important significance for affinity estimation of the different consortia of the enteric aerobic or anaerobic Bacillus spp. had dimensions of «spots» of the studied phages on the surfaces of agar cultures and the level of its transparency. Moreover we registered that some aerobical and anaerobical Bacillus spp. had common sensitivity to the same bacteriophage isolates. This observation we can explaine with presumption that we have deal with mixtures of enteric bacteriophages as well as in cases of enteric Bacillus spp. in samles of rectal swabs.

Table 1 – Results of cross-section trial profiles of enteric microbioma consorties "Bacillus spp. – Their phages" (of "host range" of the phages isolates)

No of cases	Pools of bacteriophage isolates	Bacterial lysis in pools of Bacillus spp. populations from spores of porcine rectal microbioma					
		Aerobical microbioma			Anaerobical microbioma		
		1	2	3	1	2	3
1	A_1	+	0	0	+	0	0
	B_1	+	0	0	+	0	0
2	A_2	0	+	+	0	+	+
	B_2	0	+	+	0	+	+
	C_2	0	+	0	0	0	0
3	A_3	0	0	+	0	+	+
	B_3	0	0	+	0	+	+

Designation: + – fully transparent spots at the sites of the phages pools application; + – turbid spots at the sites of the phages pools application; 0 – no spots at the sites of the phages pools application; A, B, C – rectal swabs from separate pigs of farmer's holding (№ 2) and №1 and 3 homestead piggeries. Details see in text.

The forensics application for the bacillus spores makes expert studies are not as vulnerable to the conditions of sampling, transport and storage of samples, as we can have in cases of using the reproductive active microorganisms. Moreover, this approach is allowe to simplify and reduce the cost of research by the cutting off a huge array of non-sporal microflora for trials.

Conclusions. Our experimental methodology is the perspective to establishing the origin etiological agent in range circumstances of the epidemiological-causal investigation or in the evidence collection for the criminal proceedings. This requires of further examination and possible improvement for verification of methodology – particularly regarding the reproducibility and standardization.

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References

1. US Government (2002) Public Health Security and Bioterrorism Preparedness and Response Act of 12 June 2002 [PL 107-188]. GPO, Washington DC, USA.
2. Вишняков И.Ф. Руководство по индикации возбудителей особо опасных болезней сельськохозяйственных животных в объектах ветнадзора и окружающей среды [Текст] /Лагуткин Н.А., Смирнов В.Н., Бузун А.И. [и др.] // Российская академия сельскохозяйственных наук, Всероссийский НИИ ветеринарной вирусологии и микробиологии, М.-2000, 56 с.
3. OIE Terrestrial Manual 2012. 1. CHAPTER 2.1.2. AUJESZKY'S DISEASE (http://www.oie.int/file-admin/Home/eng/Health_standards/tahm/2.01.02_AUJESZKYS_DIS.pdf)
4. Sahin M. MODERN TURKISH APPROACHES TO SOILS' DECONTAMINATION FROM ANTHRAX' AGENT WITH ATTENTION TO UKRAINE NEEDS [Text]/ Buzun A. I., Buyuk F., Celik E., Koltchik H. V., Rahimov J.// Journal for Veterinary Medicine, Biotechnology and Biosafety.- 2015- V.1.- Issue 1.- pp. 32-38 (www.jvmbbs.kharkov.ua).
5. Abshire, T.G., e.a. Production and validation of the use of gamma phage for identification of Bacillus anthracis. [Text] / Brown, J.E. and Ezzell, J.W.// J Clin Microbiol-2005 - 43, 4780–4788

УДОСКОНАЛЕННЯ ДОКАЗОВИХ ДОСЛІДЖЕНЬ: З ПРАКТИКИ СУДОВО-ВЕТЕРИНАРНОЇ ЕКСПЕРТИЗИ ХВОРОБИ АУЄСКІ СВИНЕЙ

Бузун А.І., Кольчик О.В., Бобровицька І.А., Стегній А.Б., Вовк С.І.
Національний науковий центр «Інститут експериментальної і клінічної ветеринарної медицини», м. Харків, Україна

Яценко І.В.
Харківська державна зооветеринарна академія, м. Харків, Україна

З огляду на глобальну дестабілізацію та сучасну ситуацію з поширенням африканської чуми свиней у сусідніх до України державах будь-які прояви масової захворюваності у вітчизняному свинарстві мають розглядатися у форматі судово-ветеринарної експертизи – з метою своєчасного виявлення та ліквідації наслідків. Нажаль на сьогодні в Україні недостатньо розроблено методологію збору доказів агротероризму та злочинів із застосуванням біологічних засобів (біозлочинів).

Методологію доказових досліджень (ДД) відпрацьовували на прикладі спалаху хвороби Ауєскі (ХА) у дрібнотоварному свиногосподарстві, де спалах хвороби стався після завозу ремонтного кнура. Етіологічні дослідження на ХА проводили згідно стандартних операційних процедур (СОП) ННЦ «ІЕКВМ» з урахуванням вимог МЕБ. Експериментальна схема ДД була спрямована на вивчення спорідненості кишечних спороутворюючих бацил (без уточнення видового складу) у неблагополучній (А), підозрюваній (Б) та контрольній (В) групах свиней. Для цього з ректальних квачів стандартними бактеріологічними методами виділяли спори кишечних бацил і літичні бактеріофаги. Потім пулами виділених бактеріофагів заражали пули пророщених зі спор культур кишечних бацил. Доказом спорідненості культур кишечних бацил вважали їх чутливість до одного й того пулу кишечних фагів.

За результатами досліджень розроблена методика, яка є перспективною для доказових досліджень і судово-ветеринарної експертизи хвороби Ауєскі, але потребує подальших випробувань щодо відтворюваності та рівня вірогідності.

Ключові слова: хвороба Ауєскі, судово-ветеринарна експертиза.

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ОЦІНКА ЧУТЛИВОСТІ ТА СПЕЦИФІЧНОСТІ РОЗ-БЕНГАЛ ПРОБИ ЗА ДІАГНОСТИКИ САПУ

Козій Р.В.*

Державний науково-контрольний інститут біотехнології і штамів мікроорганізмів, м. Київ,Україна, e-mail: rvkoziy@yahoo.com

Серологічні методи відіграють вирішальну роль у лабораторній діагностиці сапу. У цій статті описано визначення чутливості та специфічності РБП для серологічної діагностики сапу. Роз-Бенгал проба (РБП) – простий та швидкий експрес-тест, який можна використовувати у поєднанні з іншими методами для діагностики сапу.

Матеріали і методи. Досліджено панель з 711 польових негативних зразків сироваток коней та лабораторну панель з 94 позитивних сироваток коней. Реакцію проводили згідно інструкції виробника.

Результати. Встановлено, що чутливість РБП склала 80,9 %, а специфічність – 90,2 %. Таким чином, доцільно використовувати РБП як експрес-метод для діагностики сапу лише у поєднанні з іншими лабораторними та клінічними методами діагностики та викласти отримані результати в Інструкції щодо профілактики та боротьби з сапом тварин.

Ключові слова: сап, серологічна діагностика, Роз-Бенгал проба, РБП.

Сап (лат. malleus; англ. glanders, farcy; фр. morve; нім. Rotz; ісп. muermo) – це висококонтагіозна інфекційна зоонозна хвороба, яка викликається бактерією *Burkholderia mallei* (раніше *Pseudomonas mallei*). На сап в основному хворіють представники родини конячих. До сапу чутливі й деякі інші види тварин, зокрема верблюди, представники родини котячих та собачих, дрібна рогата худоба, а також люди. В останні роки сап набуває все більшого значення, оскільки за рахунок руху коней під час міжнародної торгівлі, міжнародних змагань тощо існує небезпека занесення хвороби з ендемічних (Бразилія, Близький Схід, Пакистан, Індія, Монголія та ін.) у вільні регіони [1].

Єдиним природним резервуаром збудника сапу у природі є коні [2]. У зв'язку з цим, своєчасне виявлення та ізоляція хронічно та латентно хворих тварин є основною передумовою ефективного контролювання поширення сапу.

Пряме виявлення B. mallei бактеріологічним чи молекулярно-генетичним методами часто ускладнене, особливо у хронічно та латентно хворих тварин, у яких слабо виражені клінічні ознаки [2].

Алергічна малеїнова проба, розроблена в 1891 році, була основним засобом діагностики сапу в СРСР, країнах Європи та Північної Америки у рамках заходів з ліквідації цієї хвороби у першій половині ХХ століття. В ендемічних регіонах основним методом діагностики сапу залишається малеїнова проба. Однак, малеїнізація може давати негативні або сумнівні результати у 10 % випадків за дослідження інфікованих коней, особливо у старих або виснажених тварин. Можлива також хибно-позитивна реакція за стрептококової інфекції [3]. Крім того, малеїнова проба викликає гуморальну реакцію та утворення специфічних антитіл, що перешкоджає наступним серологічним дослідженням [2]. У зв'язку з цим, згідно міжнародних вимог, провідне місце у лабораторній діагностиці сапу посідають серологічні методи.

Одним із серологічних методів, запропонованих для діагностики сапу є Роз-Бенгал проба (РБП). РБП – це варіант реакції аглютинації, за якої використовують антиген, зафарбований барвником Роз-Бенгалом, для кращої візуалізації реакції. На сьогодні, ця реакція валідована лише в Російській Федерації, і не рекомендується МЕБ для міжнародної торгівлі [4]. Разом з тим, у діючій

* Науковий керівник – Скрипник В.Г.