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TITLE: Methods of Virus Isolation from the Urethra of
Patients Suffering from Non-Gonococcal Urethritis

[Metody Vydeleniya Virusa iz Uretry u Bol'nykh
Negonokokkovym Uretritom.]

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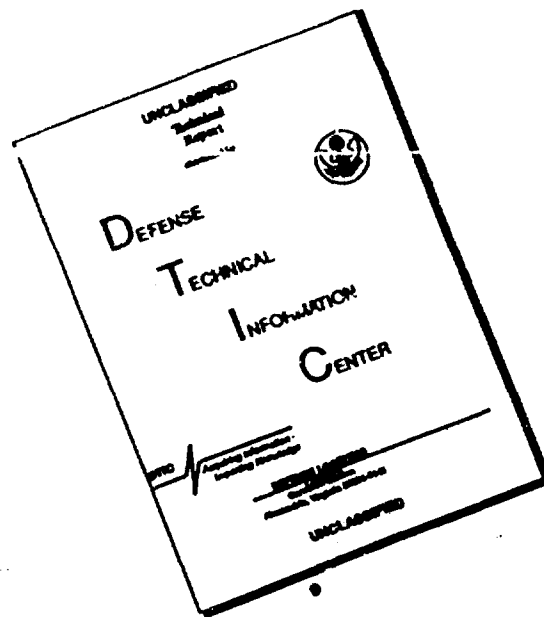
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Non-gonococcal urethritis whose pathogenic agent can be a virus is rather frequent in patients with urological ailments.

In the Institute of Poliomyelitis and Viral Encephalitides of the USSR Academy of Medical Sciences, we isolated peculiar strains of a filterable virus from the urethra of men whose wives suffered from chronic cervicitis. The separated and re-isolated pathogenic agents were small globular formations 0.2 to 0.5 microns in size, stainable after Romanovsky-Giemsa. They resembled the virus of trachoma and inclusion blennorrhoea. In addition to elementary and initial bodies, these viruses formed intracellular cytoplasmic inclusions which reached a diameter of 6 microns. They could be cultivated in the yolk sac of chick embryos, and, depending upon the passage, they caused the death of the embryos. Using various methods of infection, the agents were non-pathogenic to laboratory animals, but in monkeys they provoked follicular conjunctivitis, and cervicitis. Isolated strains of the pathogenic agent were sensitive to tetracycline, oxytetracycline, erythromycin, oleandomycin, oleotetrine, and insensitive to streptomycin, ristomycin, monomycin, and mycerin. Their morphological, biological, and serological study showed that the isolated viral strains evidently occupy a special place between the large viruses and the rickettsias, i.e., a place close to the group of the agents of psittacosis, venereal lymphogranuloma, and trachoma, although they also possess special characteristics.

Because of the dimensions, in an average of 10% of the cases, the pathogenic agent of oculogenital infection can be detected with the aid of an ordinary microscope at a viroscopy of scrapings taken from the urethra of patients with chronic cervicitis, and urethritis. In addition to this, the methods of virus isolation must be used with the aid of infecting chick embryos and providing for passage of elementary and initial bodies of the virus in the yolk sac of developing chick embryos.

The procedure of isolating the viruses in chick embryos is more effective in the diagnosis of viral urethritides than a viroscopy of scrapings. This can be explained in that in the yolk sac of chick embryos the viruses multiply rapidly, accumulate in a large amount, and are easily detected. The mode of their isolation in chick embryos can be used for studying the epidemiology of a certain infection with the examination of both men and women, and this method allows one to determine the source which maintains the circulation of virus in family foci. The method of viroscopy alone can rarely be efficient in such cases. In our practice, we observed cases where, at viroscopy of the smears taken from the urethra of men and women, viral bodies were not found, but, with the aid of chick embryos and blind passages, virus strains could be isolated. Therefore, viroscopy of scrapings must be combined with infection of chick embryos to assure a correct and timely diagnosis of viral urethritides. Moreover, by using the method of chick embryo infection, highly specific antigens can be obtained for the RSK (cytotoxic reaction) as well as for an experimental selection of chemotherapeutic agents and for a comparative evaluation of their effectiveness.

Procedures of Virus Isolation

For isolation of virus from the urethra and for its passage in the yolk sac of chick embryos, conditions are required which assure sterile work. Materials for virus isolation are urethral scrapings which are microscoped in advance for finding elementary or initial bodies, and intracellular inclusions; the cellular composition of the preparation is also determined, i.e., a cytoscopy is made. Viroscopy and cytoscopy sometimes permit the establishing of the viral nature of the urethritides. But most often, together with them, the yolk sac of chick embryos must be infected.

Urethral scrapings from men and women are prepared in as sterile a manner as possible, with small spoons according to the generally approved method. A part of the scrapings is placed on an object slide, fixed with methyl alcohol or with a mixture of Nikiforov, and stained after Romanovsky-Giemsa or after Machiavello. A larger portion of the scrapings is placed in a bottle with phosphate buffer (2 ml) containing streptomycin (500 units per 1 ml), ristomycin (125 units per 1 ml), monomycin (250 units per 1 ml), and mycerin (200 units per 1 ml). These antibiotics are used for the suppression of a concomitant bacterial flora, but they do not kill and do not weaken the virus. The inoculums are kept at -20°C for 2 months, and at 4°C up to 10 days. It is, however, desirable that the time the inoculums are kept at 4°C should not exceed 3 full days.

For an infection of chick embryos, the material taken from the patients is cultivated on meat-pepton and glucose bouillon, on Sabouraud's medium, on Kitta-Tarozzi medium, and for the detection of minoplasma (PPL0) on a semifluid 0.3% solution of Difco agar with a 10% solution of horse serum. Inoculums which are infected with any microflora from which they could not be cleared with antibiotics are not used in the experiments. 0.5 ml of the inoculums is introduced into the yolk sac of 7-8 day old chick embryos through the air space with a long needle (3-4 cm). Above the air space, in the center an aperture is made; the egg is placed on a stand, or kept in the left hand by its blunt end to the right, and through the formed aperture, in a vertical direction toward the center of the egg, the needle is introduced, with the syringe, which contains the viral material attached to it. In advance, at the transillumination of the egg, the boundary of air space and yolk sac are noted. The aperture is sealed with paraffin; the embryos are placed in a thermostat at 35°C , and transilluminated daily.

Embryos which died in the first three days are rejected from the experiment, since their death is considered non-specific (due to bacterial infection, injury). Only from the 4th to the 5th day on can the death of embryos be specific for the virus. The autopsy of embryos and the withdrawal of yolk sacs occurs under sterile conditions. The egg-shell above the air space is disinfected with alcohol and iodine, and cut away with sterile scissors at the boundary of the air space. Then, the putamen and the chorioallantoic membrane are incised; the embryo is withdrawn with a forceps; the umbilical stalk is resected; the yolk sac is separated and placed in a separate sterile bottle containing penicillin. The sterility is checked, and the presence of viral particles in the yolk sac is verified. Every yolk sac is subjected to this procedure, before it is used for further experiments, i.e., before the production of a subsequent passage. Suspensions of 20 to 50% are prepared from the chick embryos which had been infected with the virus of yolk sacs, and either died after the third day of incubation, or remained alive, provided that elementary bodies were observed in them, and they showed no bacterial contamination. A phosphate buffer with antibiotics was used as a solvent. After a bacteriological checkup, the obtained suspension of yolk sacs was introduced into a batch of new eggs.

Preparation of Smears and Imprints
for Virosopic Examination and Cytoscopy

Smear imprints are prepared by applying small pieces of yolk sac to object slides. The imprints are fixed with methyl alcohol, or with Nikiforov mixture, for 3 to 5 minutes, and stained after Romanovsky-Giemsa. For this purpose, a ready solution is available for sale; it contains the Romanovsky-Giemsa stain at the rate of 2-3 drops per 1 ml of distilled water. The imprints, just as the scrapings, are

stained with this solution for 1 to 2 hours at room temperature, or with a boiling solution of the Romanovsky-Giemsa stain (5-10 drops per 1 ml of distilled water) for 5-8 minutes. After staining, the preparation is differentiated with 96° alcohol for 1-2 seconds, and washed with tap water. After drying, the imprint can be used for microscopy. According to Machiavello's method, the scraping or imprint of the yolk sac is stained with a 0.25% solution of basic fuchsin for 5 minutes; then it is dried gently, and transferred for a few seconds into a 0.5% solution of citric acid; after which it is washed with tap water, and additionally stained with a 1% solution of methylene blue for 20-30 seconds, then washed with water and dried.

Intracellular inclusions are usually stained in dark-blue color; cell nuclei are pale rosy color. The initial bodies are most often bright blue, and the elementary bodies a reddish color which is at the limit of visibility with an ordinary microscope.

Cultivation and Passage of the Virus

As it was shown above, in absence of a bacterial contamination and in presence of the virus in the yolk sacs, the sacs are used for the preparation of suspensions. In such cases, when, in the absence of a contamination with microflora, virus cannot be found in the smear imprints, blind passages must be made. If, after 5-6 passages, virus cannot be isolated at all, the result may be considered negative.

The frequency of virus finding can be influenced by the length of cultivation. Therefore, if there is no death of embryos, we should not hurry with the autopsy sooner than the 12th day of incubation after infection.

Virus can be isolated from the initial material also, but most often in subsequent passages. For passing the pathogenic agent, virus-infected yolk sacs, free of bacterial flora, are

taken, and placed in bottles with beads with the aid of which, by thorough shaking, suspensions are prepared. Most often, a phosphate buffer of pH 7.0 with antibiotics (streptomycin, ristomycin, monomycin, and mycerin) or physiological saline are the solvents. The suspensions are centrifuged in advance at 1000 r.p.m. for 10 minutes, and the middle layer is used.

For passage, various doses of the virus-containing suspension are used, depending upon the virus titer, the conditions, and the duration of its storage, and so on. The virus titers are determined on chick embryos by their specific death, or morphologically by the imprints of yolk sacs with the virus bodies. They can reach a dilution of 10^{-7} in 0.5 ml of a yolk-sac suspension. The morphological method of virus titer determination is more reliable and more accurate than the specific death method since sometimes a seasonal resistance of chick embryos can be observed to a certain virus, and also in the percentage of deaths of chick embryos a variability is noted according to the passage. Frequently in the yolk sacs of surviving chick embryos many elementary viral bodies can be found, while no specific death is observed.

Evaluation of the Results of Virus Isolation

Results are evaluated not only by the discovery of viral particles in the urethral scrapings, but chiefly on the basis of the appearance of elementary particles, of initial bodies, and intracellular inclusions of the virus in smear-imprints of yolk sacs, as well as by the specific deaths of infected chick embryos from the 4th on to the 12th day after infection.

Morphologically, the virus which is present and which propagates in the yolk sac of chick embryos, bears much resemblance to the virus particles and intracellular inclusions which can be detected at the viroscopy of urethral scrapings

in which usually cellular changes are constantly noted.

These cellular changes appear as abnormal epithelial cells with vacuolization, disintegration in the cytoplasm; sometimes polymorpho-nuclear leukocytes, lymphocytes, plasma cells, and mononuclear cells can be found.

These cellular changes can be an indirect sign of virus infection, if virus was not found in the initial material, since in presence of specific cellular changes a direct isolation of the virus is not always successful. Sometimes a few subsequent blind passages are required for this.

An active multiplication of the virus in the yolk sac is also proved by hyperemia, a marked thinning of the yolk-sac wall, and liquefaction of its contents. At microscopy of yolk-sac imprints in this period, together with unchanged cells, edematous or hypertrophic cells can also be found, and cells containing the virus in various stages of its development (intracellular as well as extracellular forms). Moreover, increase in basophilia, and in vacuolization of the cytoplasm is noted, and vacuolar degeneration of the nuclei can also be met with quite often.

In initial stages of viral development, viral particles of larger dimensions (0.4-0.5 micr.) and compact inclusions (up to 10 micr.) predominate; in the subsequent stage, their size decreases (0.2-0.3 micr.), and the intracellular inclusions do not exceed 6 microns.

In evaluating the morphological changes caused by the virus in the yolk sac, the possibility of detecting micrococci, PPLO, and so on, should be kept in sight, which can be distributed intracellularly, and can cause changes in cells. In such cases, with the aid of sowings of yolk-sac pieces on meat-pepton bouillon, on blood media, on semifluid Difco agar with horse serum, and so on, a bacteriological contamination can be detected easily, although even without sowings experienced

investigators can distinguish morphological changes in yolk-sac cells which were caused by the virus, and changes which were brought about by bacterial and other non-specific causes.

Only by way of a comparison of all laboratory data can an evaluation of the results be correct.

Summary

The most rational method for virus isolation from the urethra, for propagation and passage of the virus in the yolk sac of the developing chick embryo is presented. The results of virus isolation are evaluated, and some recommendations for laboratory diagnosis of viral urethrites are given on the basis of personal experience of work with this infection. This method may find a wide application in practice of examination of patients with non-gonococcal urethrites in urological clinics.