Ova prezentacija objedinjuje bakteriologiju i virologiju. Problematika, poznata kao „*Phage Therapy*” predstavlja aktualni hit u području koje nazivamo „*Life Sciences*”.

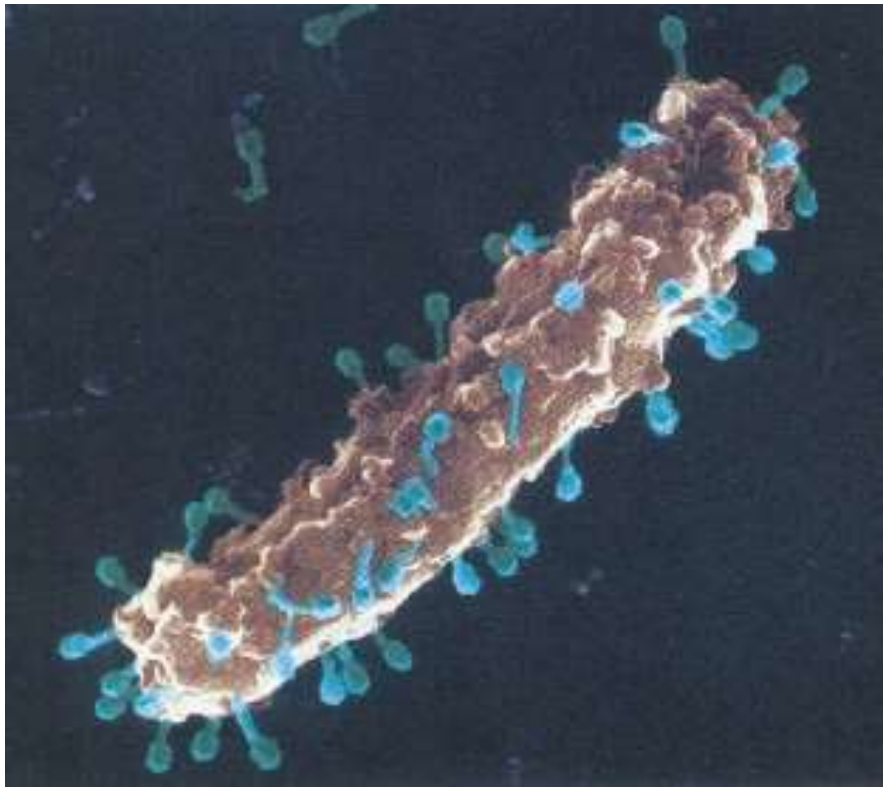
Prezentacija je pripremljena kao dio *Virologije* u okviru međunarodne ljetne škole (2020) na *Sichuan University Chengdu* (zbog pandemije koronavirusa nije održana).

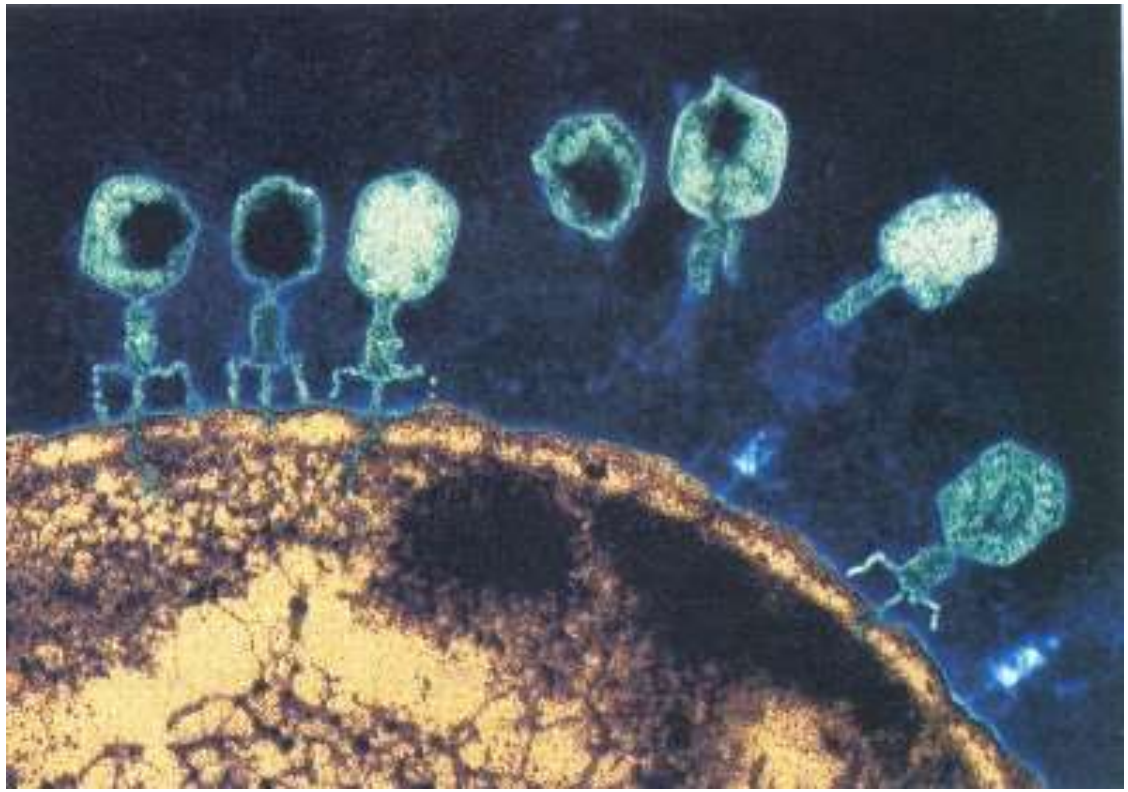
Prezentacija započinje povijesnim činjenicama o otkriću bakterijskih virusa te pionirskim pokušajima da ih se primijeni u borbi protiv patogenih bakterija. Spominje se otkriće antibiotika koji su na neko vrijeme skrenuli pozornost s bakteriofaga, a potom upozorava na bakterijsku otpornost prema antibioticima i nužnost da primjenu virusa ponovno razmotrimo kao spasonosno rješenje u borbi protiv bakterijskih bolesti.

U drugom dijelu prezentacije možete doznati o osnovnim postupcima pri izolaciji virusa koji trebaju poslužiti kao terapeutici u tretmanu bakterijskih infekcija. Također, ukratko je prikazan sadržaj znanstvene publikacije s opisom kliničkog slučaja u kojem se spašava život čovjeka koji treba umrijeti zbog sepse uzrokovane rezistentnom bakterijom.

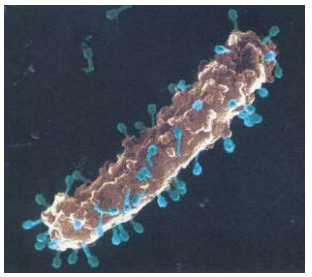
Prezentacija će vam pomoći da shvatite zašto su bakterijski virusi opet „u modi”.

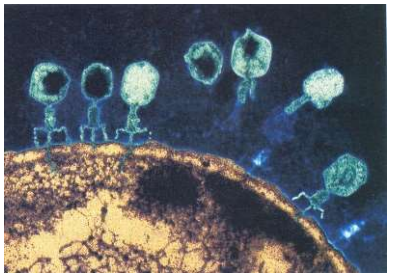
Bacteriophage therapy





bacteriophages attached
to cell wall of *Escherichia coli*





In 1896 a British bacteriologist Ernest Hankin reported on the presence of marked antibacterial activity against *Vibrio cholerae* which he observed in the waters of Ganges river in India. Twenty years later, Frederick Twort, a medically trained bacteriologist from England, was the first who has thought that the phenomenon may have been due to viruses. As Twort did not publish that hypothesis, another two years past before bacteriophages were „officially" discovered by Felix d'Herelle, a French-Canadian microbiologist at the Pasteur Institute in Paris. It was associated with an outbreak of severe hemorrhagic dysentery among French troops in the Word War I (2015). D'Herelle made bacterium-free filtrate of the patients' fecal samples, mixed it with *Shigella* strains isolated from the patients, and cultivated in Petri dishes. It was on these agar cultures that d'Herelle observed the appearance of small clear areas, which he called **plaques**. The finding was presented in 1917 at the Academy of Sciences meeting, and subsequently published in the proceedings. In contrast to previous investigators, D'Herelle had little doubt about the nature of the phenomenon, and he proposed that it was caused by a virus capable of parasitizing bacteria. It was him again, who proposed the name **bacteriophage** (from Greek *bacterium* and *phagein*, implying that phages eat bacteria).


Plaques from a virus isolated from a compost heap near UCLA. The bacterium *is Mycobacterium smegmatis.*

Not long after his discovery (1919), d'Herelle used phages to treat dysentery. It was probably the first attempt to use bacteriophages therapeutically. The phage preparation was ingested by D'Herelle himself, the hospital's chief of clinic, and several hospital interns in order to confirm its safety before administering to a 12-year-old boy with severe dysentery. The patient's symptoms ceased after a single administration, and the boy fully recovered within a few days.

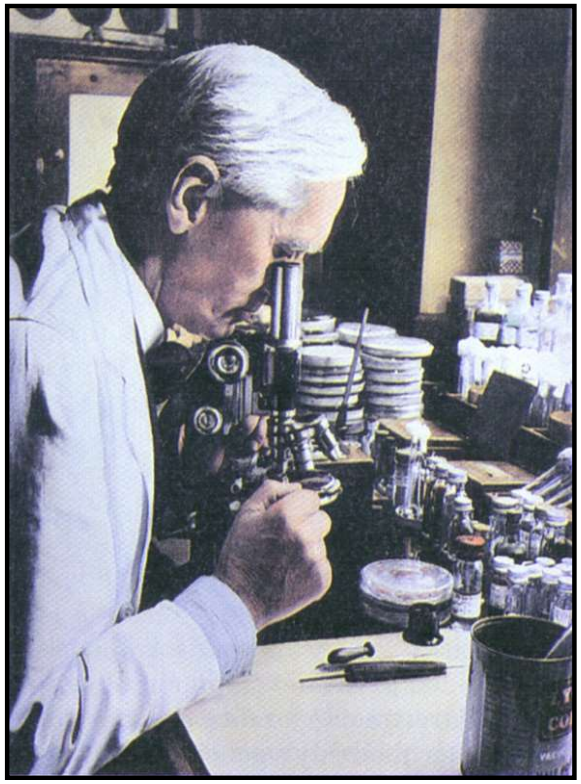
The efficacy of the phage preparation was confirmed shortly afterwards, when three additional patients, having bacterial dysentery and treated with one dose of the preparation, started to recover within 24 h of treatment.

However, the result of this study was not immediately published and, therefore, the first reported application of phages to treat bacterial diseases of humans came in 1921 (phages were used to treat staphylococcal skin disease).

D'Herelle's commercial laboratory in Paris produced at least five phage preparations against various bacterial infections. D'Herelle used phages to treat thousands of people having cholera or bubonic plague in India. Several companies in France and United States began commercial production of phages agains various bacterial pathogens.

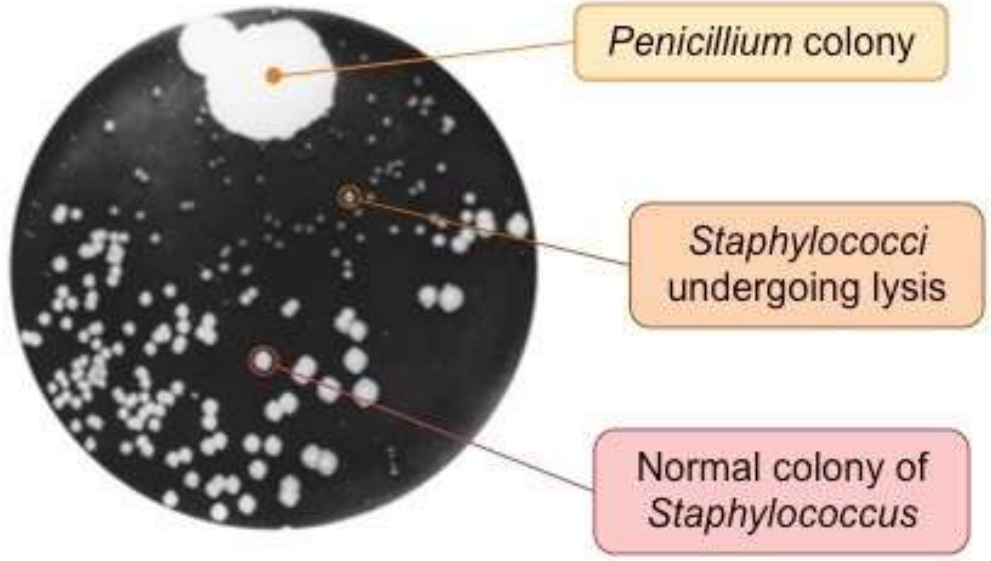
However, the efficacy of phage preparations was contraversial. Moreover, both pharmacokinetics and pharmacodynamics have not been investigated much.

With advent of antibiotics,
commercial production of therapeutic phages ceased
in most of the Western world.

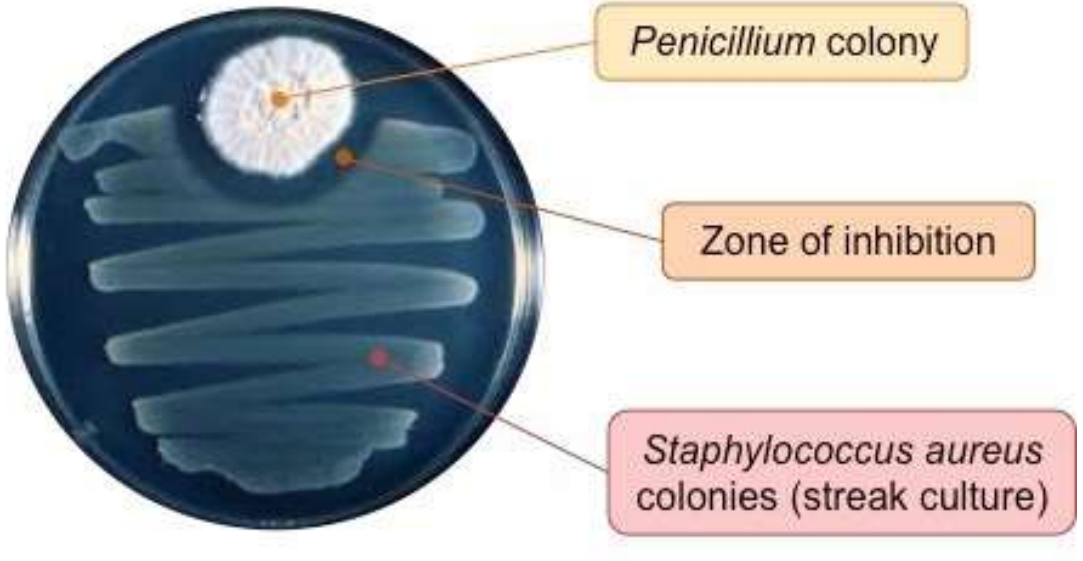


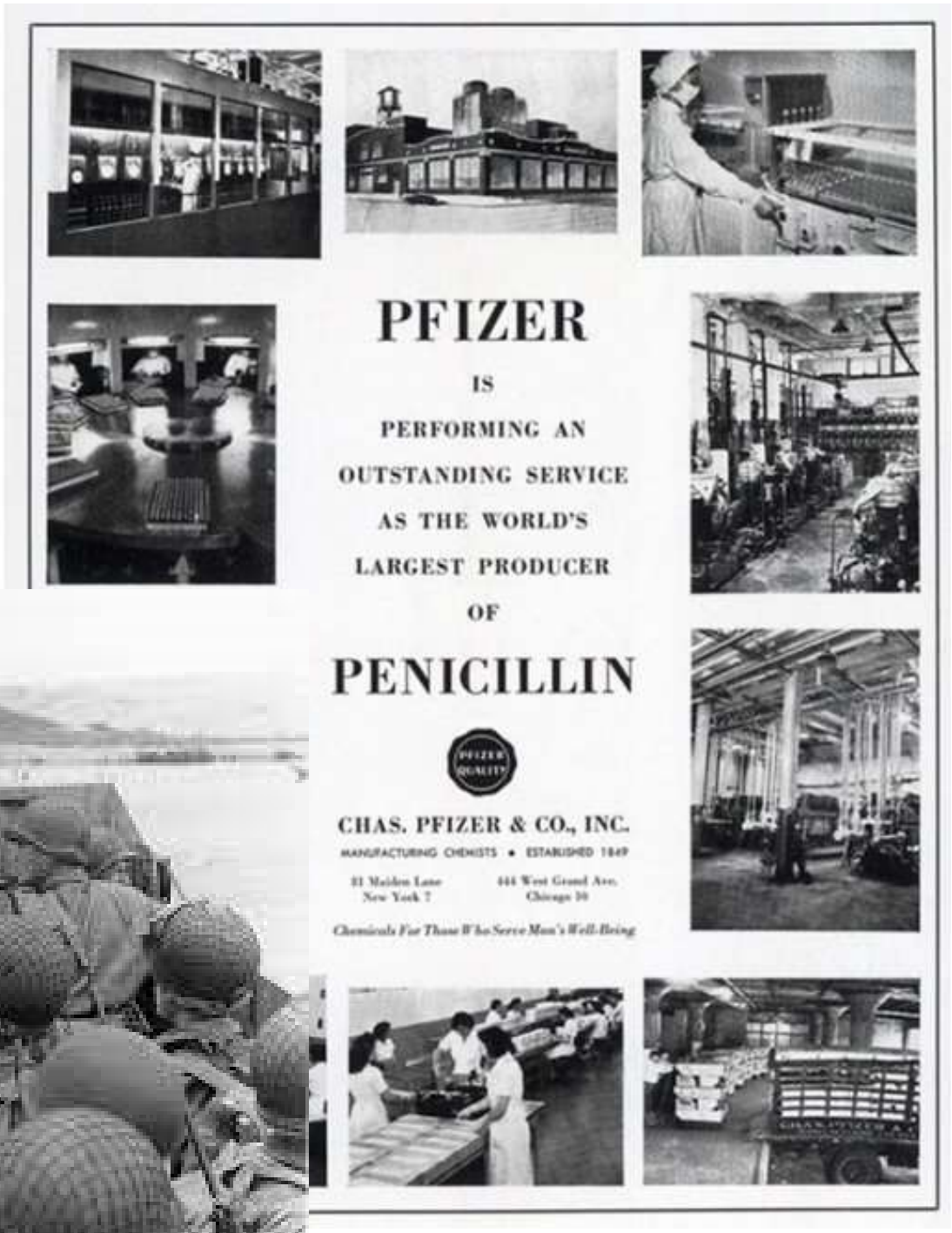
- discovery and development of penicillin (Alexander Fleming, 1928)
- isolation of penicillin (Howard Florey and Ernst Chain, 1939)

Original Experiment (Fleming, 1928)



Modern Experiment (Streak Culture)

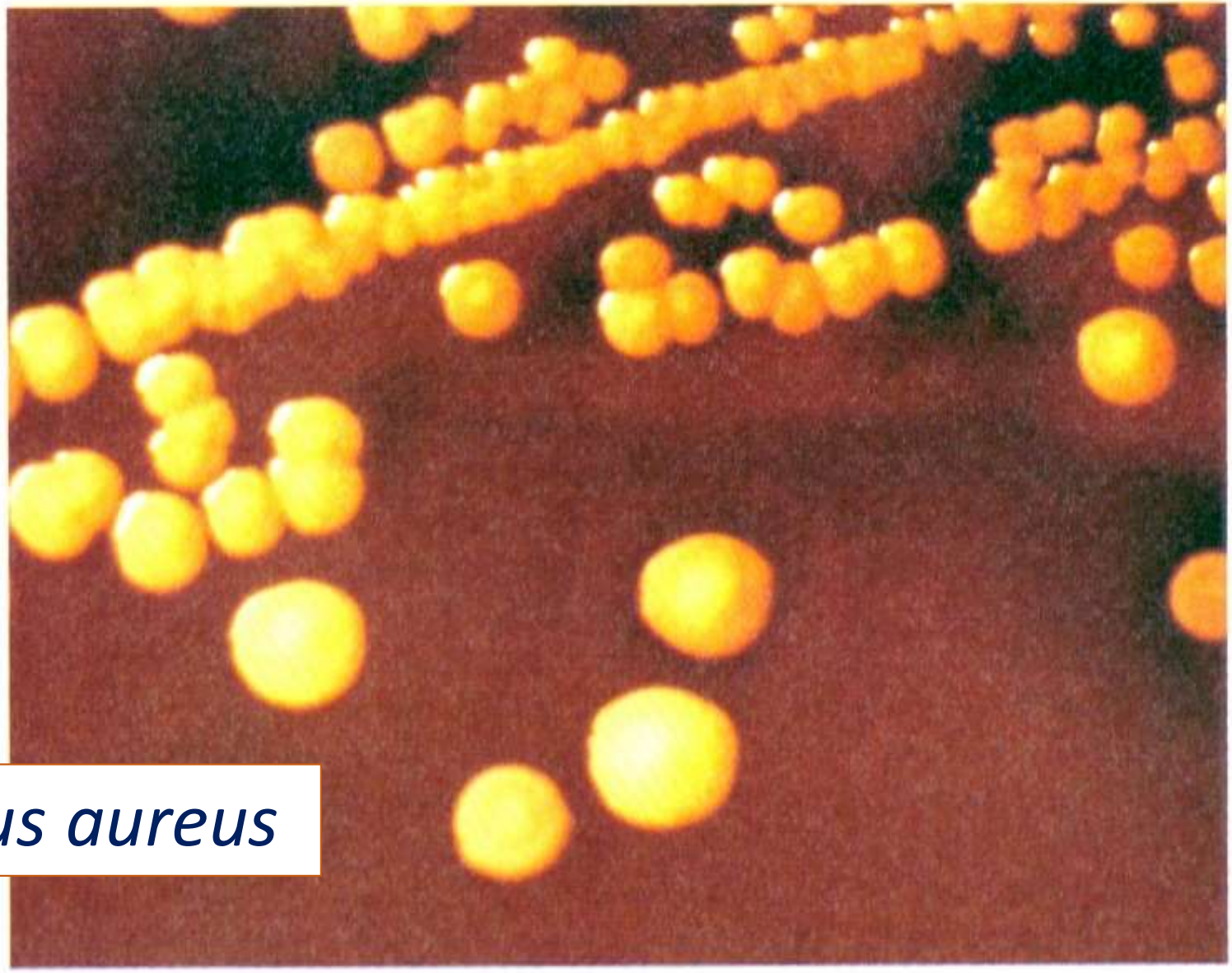




D-Day (the Normandy Invasion in 1944

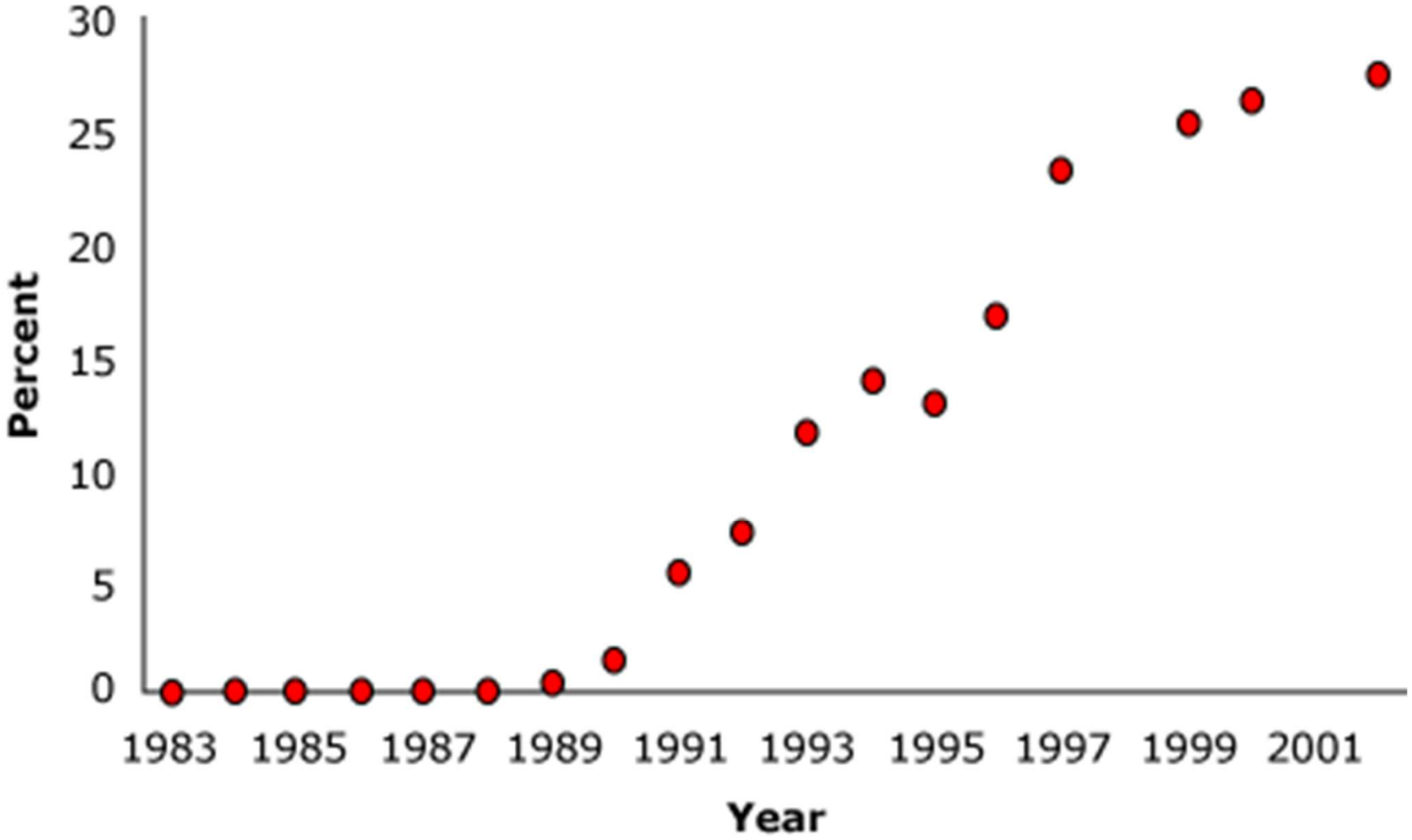
Alexander Fleming, Howard Florey, Ernst Chain
(1945) Nobel Prize in Physiology or Medicine

- Prva bakterijska vrsta na kojoj je zamijećeno djelovanje antibiotika
- Prva bakterija na kojoj je zamijećena otpornost na antibiotik (ubrzo nakon otkrića antibiotika)
- Prva bakterija koja je stekla status „*superbug*” (otporna na većinu uobičajeno korištenih antibiotika)



Staphylococcus aureus

Resistance to the antibiotic Vancomycin rose dramatically over the 1990s in US hospital intensive care units.



World Health Organization

WHO releases its first report on global antibiotic resistance (2017)

Nowadays, **increased antibiotic resistance is an important global issue.**

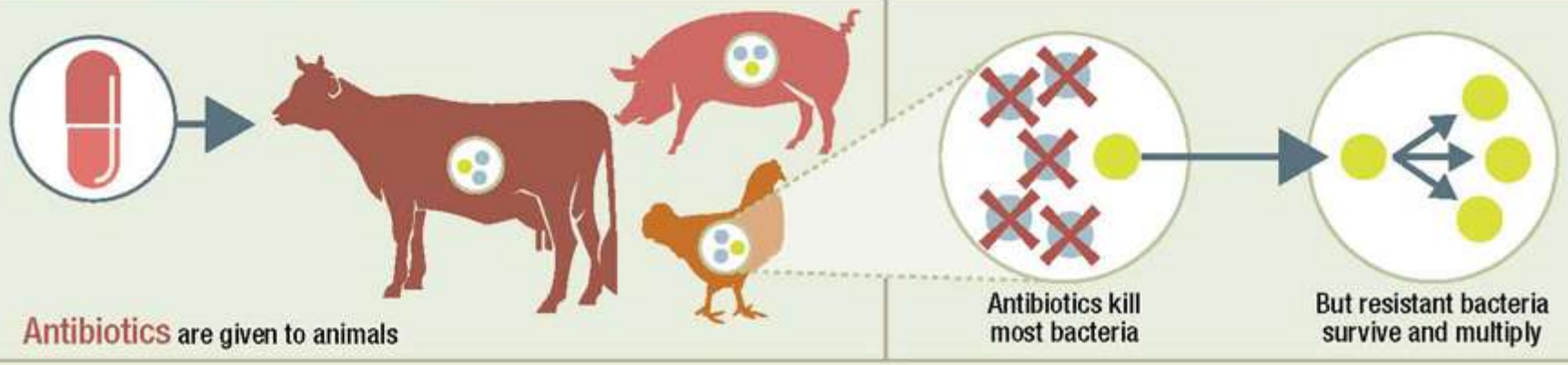
The World Health Organisation (WHO), as well as American Centers for Disease Control, and National Institutes of Health have tried to draw public attention to the growing crisis. They all have termed the present time **the "postantibiotic" era** because resistance to almost every available antibiotic exists, and multi-drug-resistant (MDR) infections are increasingly more common.

This problem stems from a variety of factors, including widespread agricultural use of antibiotics, inappropriate prescription of antibiotics, a decrease in the number of new antibiotics entering the market, and the increased positive selection of multidrug resistance when gained through the natural prokaryotic exchange of genetic material.

ANTIBIOTIC RESISTANCE

from the farm to the table

RESISTANCE All animals carry **bacteria** in their intestines

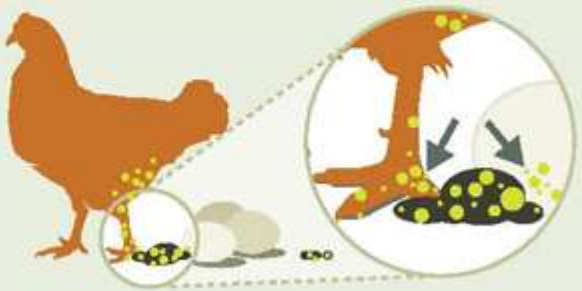


SPREAD Resistant bacteria can spread to...


animal products


produce through contaminated water or soil


prepared food through contaminated surfaces


the environment when animals poop

World Health Organization

WHO recommends that farmers and the food industry stop using antibiotics routinely to promote growth and prevent disease in healthy animals

WHO has launched new guidelines on the use of medically important antimicrobials in food-producing animals, recommending that farmers and the food industry stop using antibiotics routinely to promote growth and prevent disease in healthy animals. These guidelines aim to help preserve the effectiveness of antibiotics that are important for human medicine by reducing their use in animals. In some countries, approximately 80% of total consumption of medically important antibiotics is in the animal sector, largely for growth promotion in healthy animals.

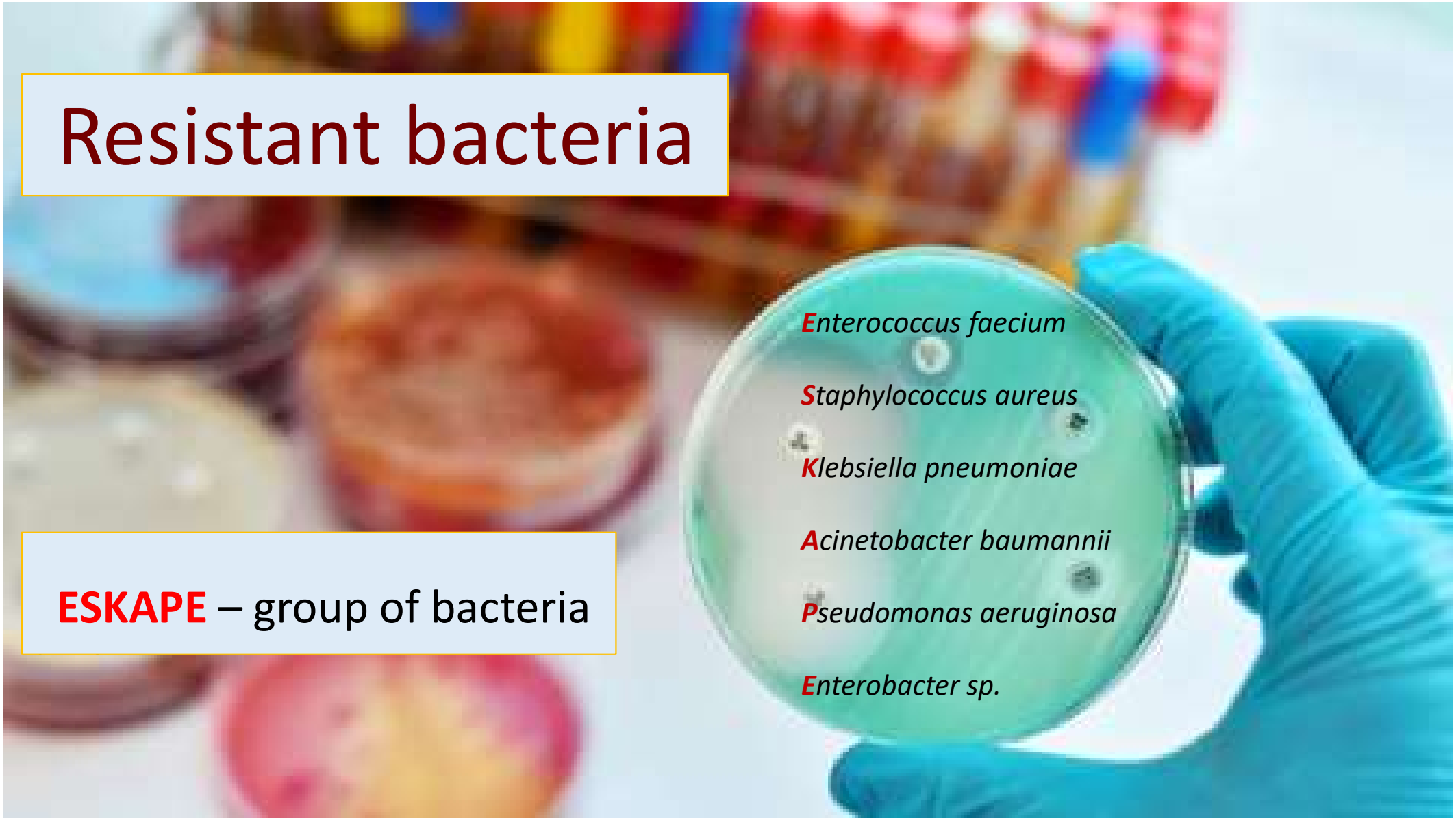
IMPLEMENTATION OF THE GLOBAL ACTION PLAN ON ANTIMICROBIAL RESISTANCE

World Health Organization

During World Antibiotic Awareness week, 13-19 November **2017**, WHO and partners reached out to the general public, health professionals, governments, farmers, veterinarians, the food and feed industry and others via a social media campaign using infographics, quizzes, and success stories to raise awareness of the need to act on antibiotic resistance and what kinds of steps we can take. The WHO, FAO, OIE World Antibiotic Awareness Week interactive online platform takes you on a unique experience to learn about antibiotic resistance. The global campaign map will be available year round for people, communities and organizations all around the world to share their activities relating to antibiotic and antimicrobial resistance. New infographics on “Did you know that superbugs can be found in food” were also made available in English, French & Spanish. In addition, “Guidelines for the prevention and control of carbapenem-resistant Enterobacteriaceae, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in health care facilities” were also released.

Resistant bacteria

ESKAPE – group of bacteria



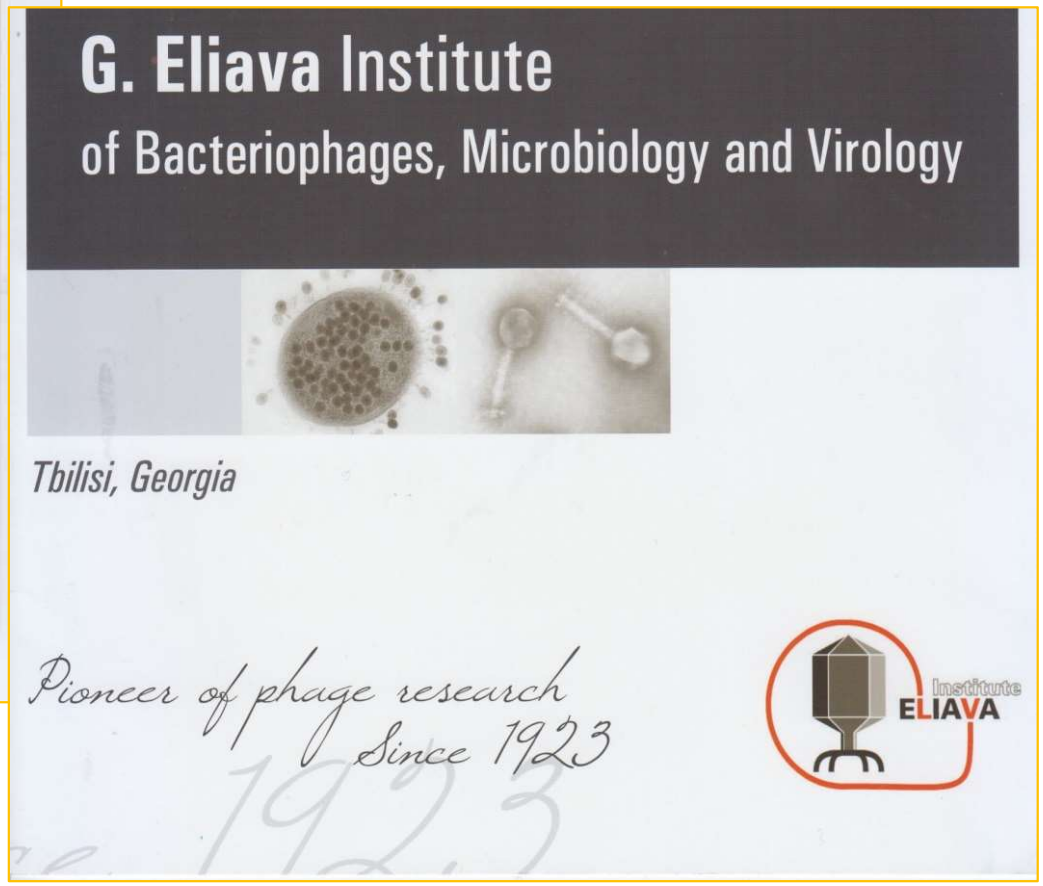
the “postantibiotic” era (since 2017)

Caused by a variety of factors:

- widespread agricultural use of antibiotics
- inappropriate prescription of antibiotics
- a decrease in the number of new antibiotics entering the market
- the increased positive selection of multidrug resistance (gained through the natural prokaryotic exchange of genetic material)

Phages continued to be used therapeutically – together with or instead of antibiotics – in Eastern Europe. Several institutions, among which Eliava Institute of Bacteriophage (Tbilisi), and Hirszfeld Institute of Immunology (Wroclaw) are the most important and generally well known.

Pioneers of Phage research:
George Eliava, Felix D'Herelle and Helen Makarashvili, Tbilisi, 1934



Pripravak virusa koji se bez recepta može kupiti u ljekarni Instituta Eliava u Tbilisiju. Namijenjeni su za površinsku i peroralnu terapiju različitih bakterijskih infekcija.

CBS News 2002
SILENT KILLERS – FANTASTIC PHAGES
Bacteria Eaters

After breaking his foot, Toronto bass player Alfred Gertler got an infection that antibiotics couldn't cure. Doctors told him he might have to have his foot amputated.

He read about a radically different way to treat infections. It was in the former Soviet Republic of Georgia, at the Eliava Institute in Tbilisi.

The treatment used something called bacteriophage – harmless viruses that have only one purpose – to eat bacteria.

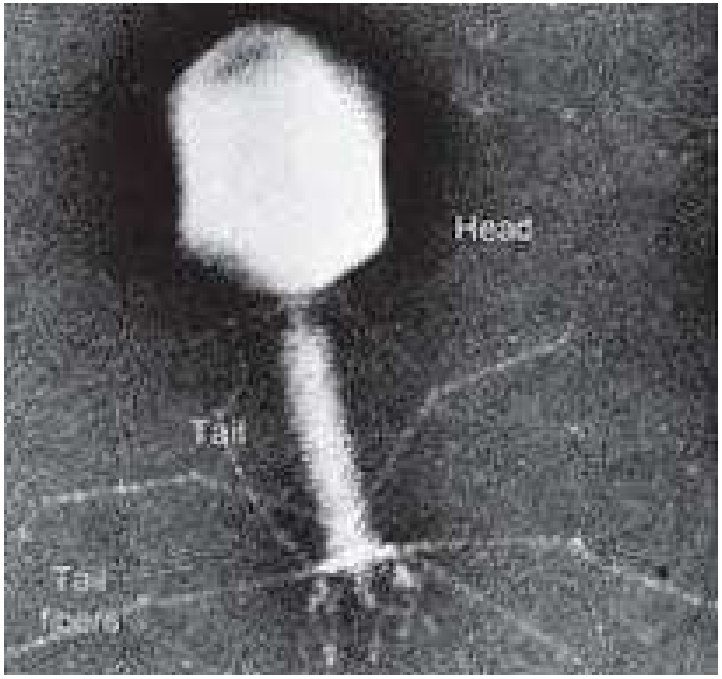
Within three days, the infection was gone.

A miracle of nature is that there seems to be a bacteriophage for every kind of bacteria. You just have to isolate bacteria and then search for the specific phages that kill them.

"We isolate the new phage from sewage!" says Mzia Kutateladze, a senior scientist of the Eliava Institute.

new treatment options are needed

Bacteriophages have re-emerged as candidates for new antibacterial therapeutics



The prevalence of multi-drug-resistant bacterial pathogens is a growing threat to public health, and it is increasingly obvious that **new treatment options are needed**.

Bacteriophages have re-emerged as candidates for new antibacterial therapeutics.

The host range of a particular bacteriophage **is often very specific** to the subspecies level. It is considered an advantage over antibiotics – **infectious bacterium can be targeted without damaging symbiotic members of the human microbial community.**

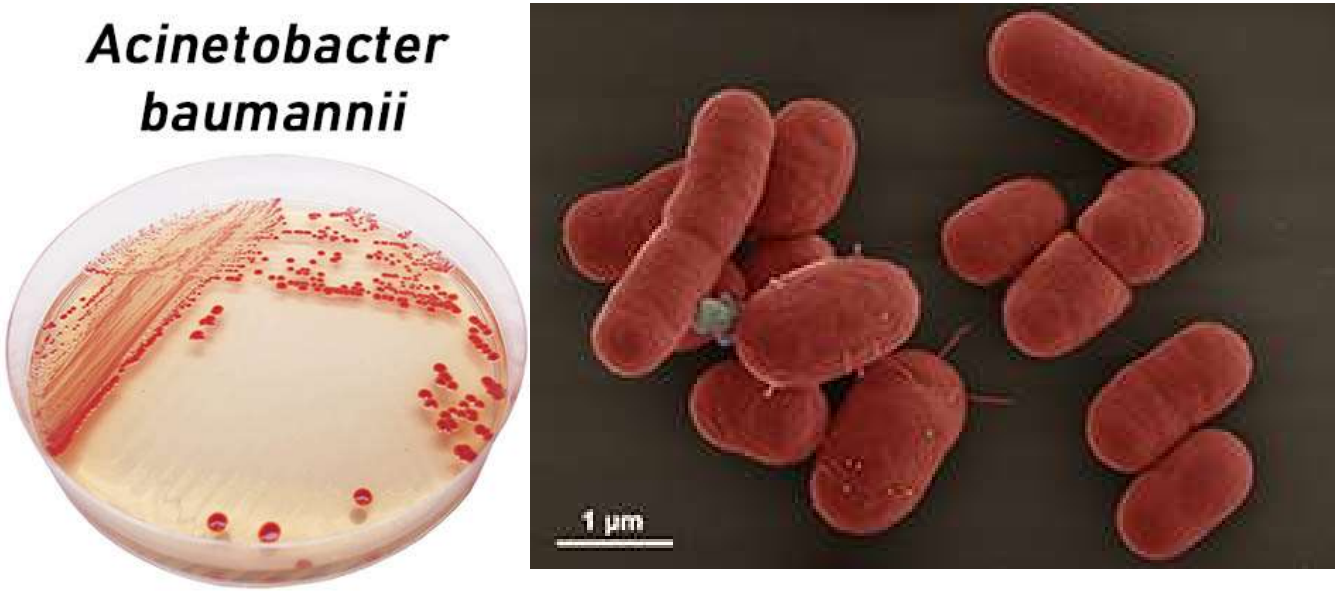
At the same time, that makes a phage therapy more complex comparing to antibiotic treatment. In principle, **phage therapeutics require a significant level of personalisation**.

As **resistance to a phage** can also occur, it is highly advantageous to prepare multiple phages against a pathogen. Combining multiple phages into a single treatment-cocktail may extend the utility of a therapy and reduce the frequency of phage resistance and therapeutic failure.

Acinetobacter baumannii is a **Gram-negative** coccobacillus. It has became important as a hospital-derived (nosocomial) pathogen. Some species of the genus are often found in soil samples.

A. baumannii is referred to as an ESKAPE pathogen, a member of a group of pathogens with a **high rate of multidrug resistance**, responsible for the majority of **nosocomial infections**. This bacterium can cause variety of diseases, ranging from pneumonia to serious blood and wound infections

Drug-resistance in this bacterial species is based on the activity of **two major afflux pumps** (pumping antibiotics out of the cell). **Horizontal transfer of antibiotic resistance genes** is responsible for spreading of the resistance.



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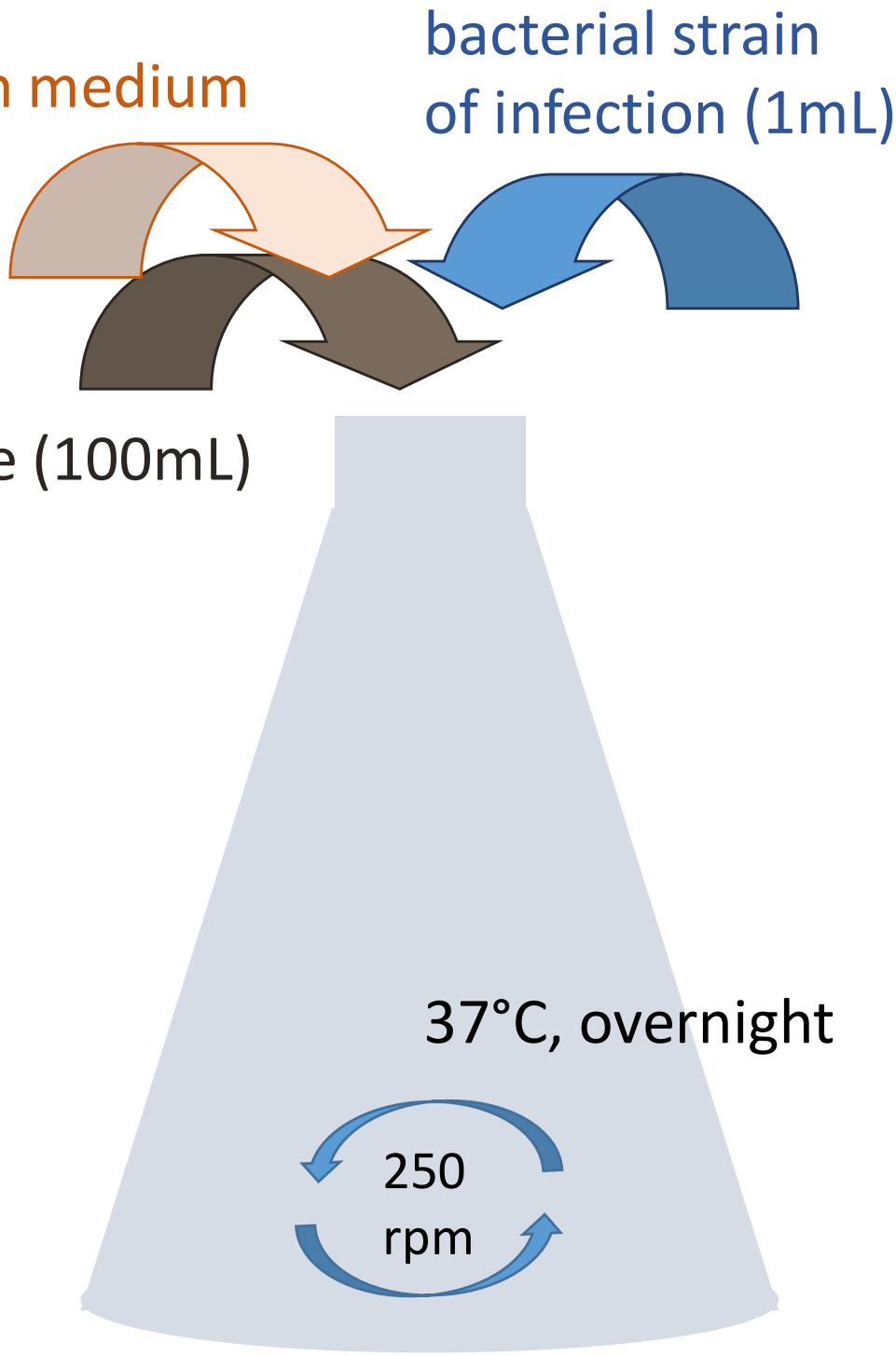
Antimicrob Agents Chemother. (2016) 60(10): 5806–5816.

In the work, presented here, several wild environmental phages against *A. baumannii* were successfully isolated and purified. It was demonstrated that such phages can be rapidly compounded into a cocktail that successfully treats *A. baumannii* wound infections in mice.

The isolation and purification of these phages from local sewage water show that phages with lytic activity against a clinically relevant pathogen can be easily and rapidly isolated from environmental sources. That would be a principle of rapid personalized phage therapy formulation.

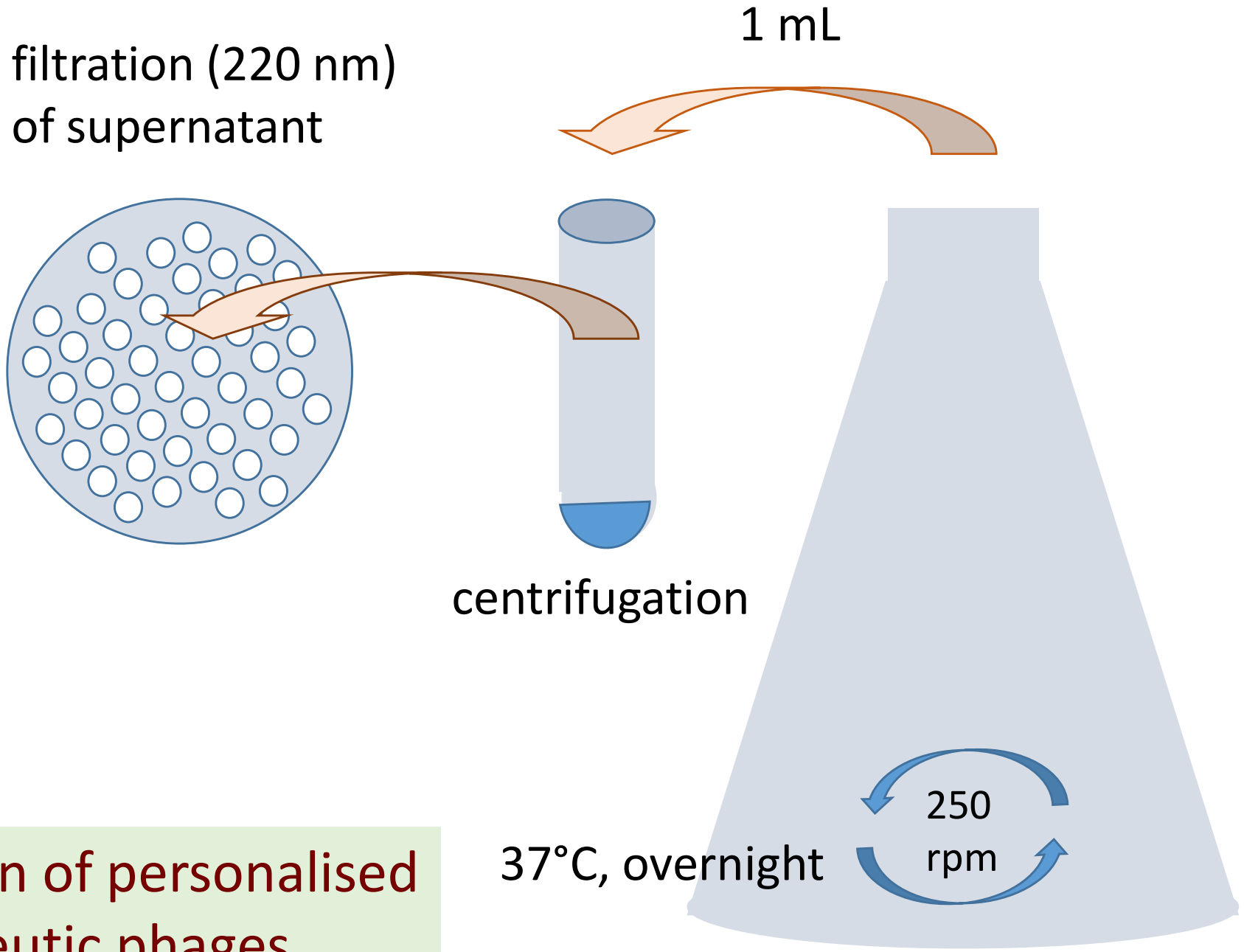
A. baumannii isolates used for phage isolation were not cured of any potential prophages first, as is customary when propagating phages in a laboratory setting. Although contaminating lysogenic viruses may be present in these phage preparations, the lytic activity of the therapeutic phages is not hindered (lysis *in vitro* and efficacy *in vivo* still occurred). Any clinical bacterial isolate could have unknown prophages as well, and taking time to cure prophages from these strains would prevent rapid formulation of a personalised phage cocktail.

Isolation of personalised therapeutic phages



Powdered TBS nutrition medium was mixed with raw sewage to a final concentration of 3% (wt/vol). Bacterial isolate was grown to exponential phase, and 1 mL was added to 100 mL of the previous mixture. That was incubated at 37°C and 250 rpm overnight.

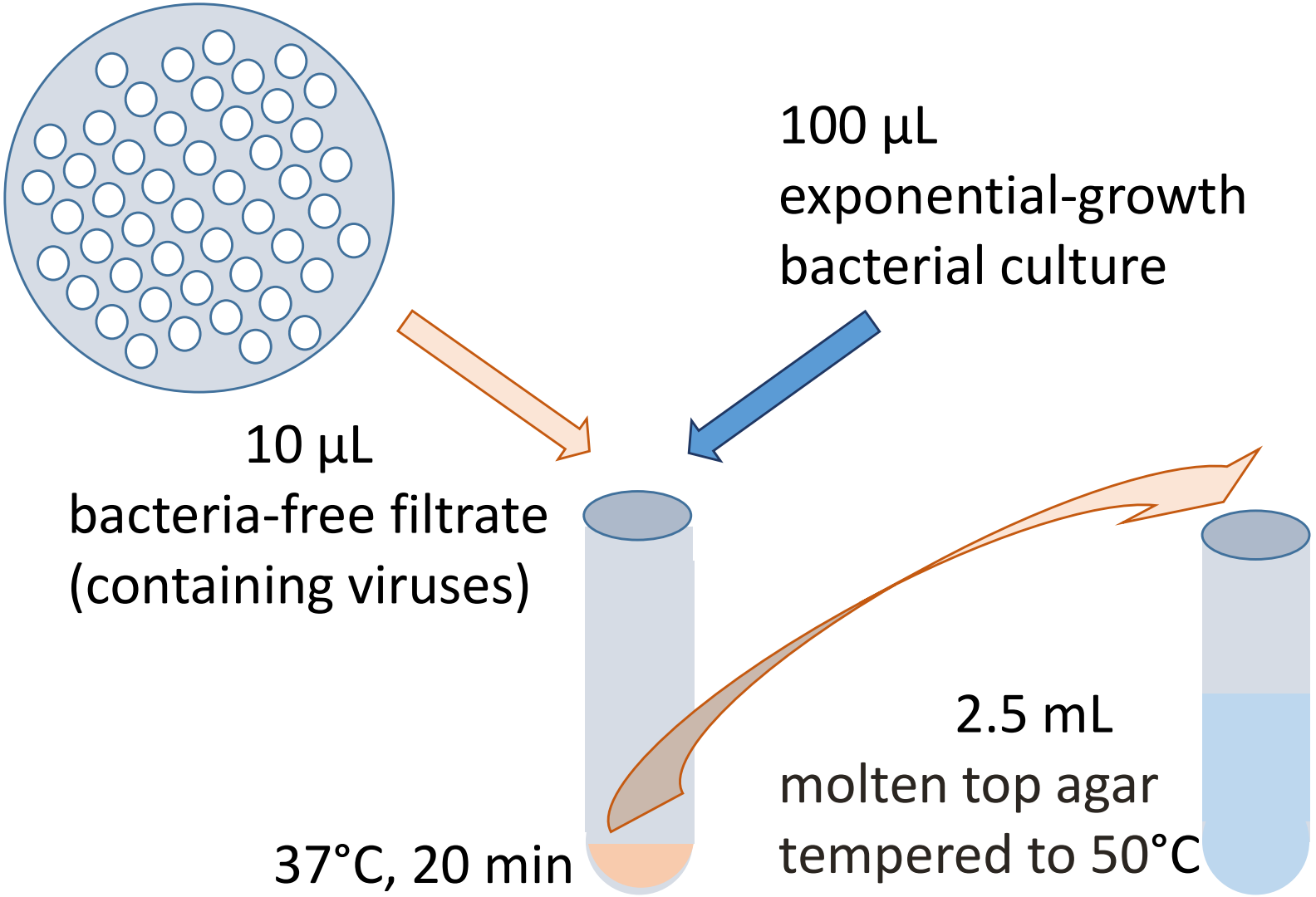
1 mL of the mixture was harvested and centrifuged (8000g, 5 min) to pellet cells and debris. The supernatant was transferred to a sterile centrifuge filter-tube (220 nm) and centrifuged at 6000g to remove any remaining bacteria.



Isolation of personalised therapeutic phages

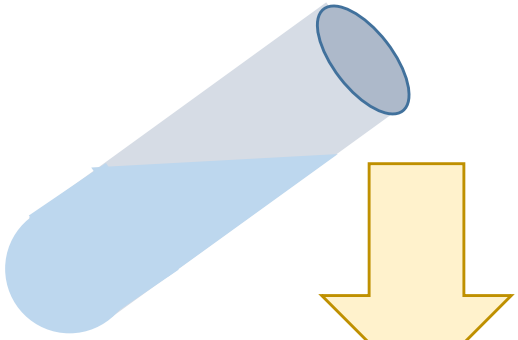
Isolation of personalised therapeutic phages

A 10 µL aliquot of the filtrate was mixed with 100 µL of exponential-growth bacterial culture, incubated at 37°C for 20 min, mixed with 2.5 mL of molten top agar (0.6% agar), tempered to 50°C, and poured over growth-medium-agar plate (1.5% agar TBS agar).



0.6% top agar
with bacteria and viruses

The plates were incubated overnight at 37°C, and subsequent phage plaques were individually harvested and purified three times on appropriate *A. baumannii* isolates.



Isolation of personalised therapeutic phages

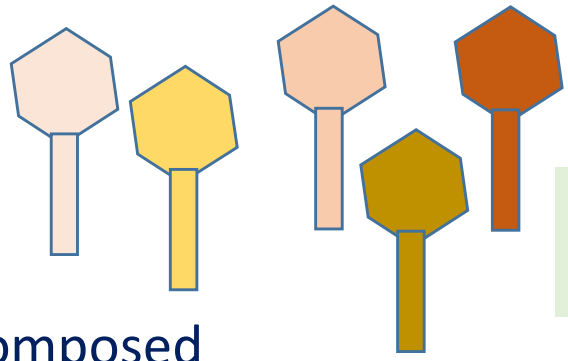
agar plate

37°C overnight

bacterial lawn

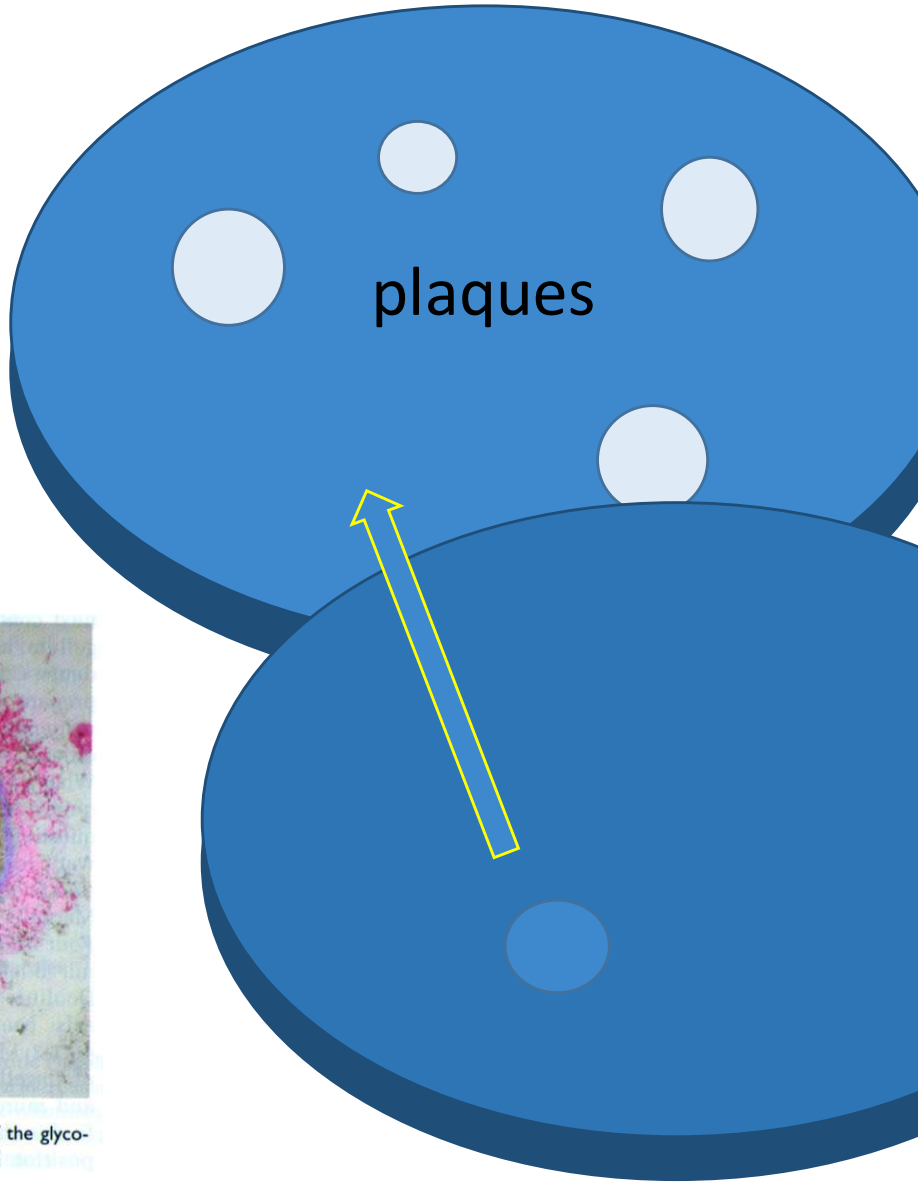
1.5% agar
with growth medium (TBS)
in a Petri dish

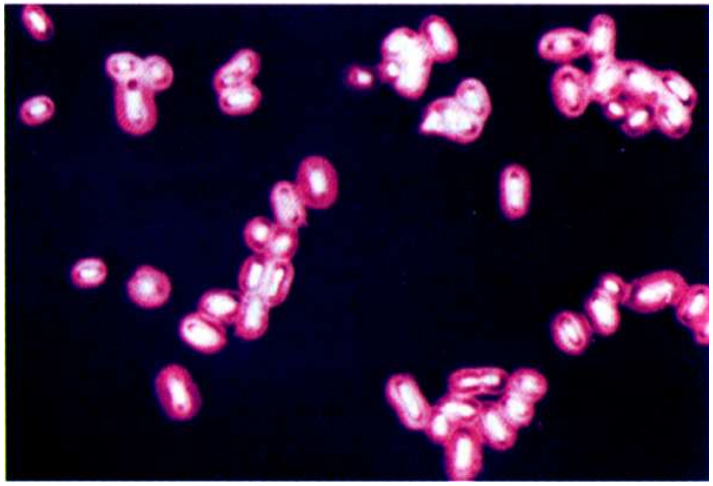
five lytic bacteriophages specific to the bacterial strain of infection



Isolation of personalised therapeutic phages

Interestingly, this effective cocktail is composed of four phages that do not kill the strain of infection, and one phage that just delays bacterial growth in vitro. A cocktail appears to function in a combinatorial manner, as one constituent phage targets capsulated *A. baumannii*, shifting the bacterial population to an uncapsulated state sensitised to the remaining four phages in the cocktail.





capsule (glycocalix)

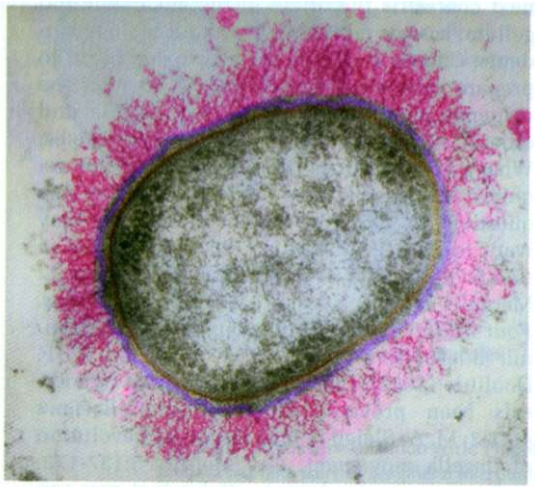
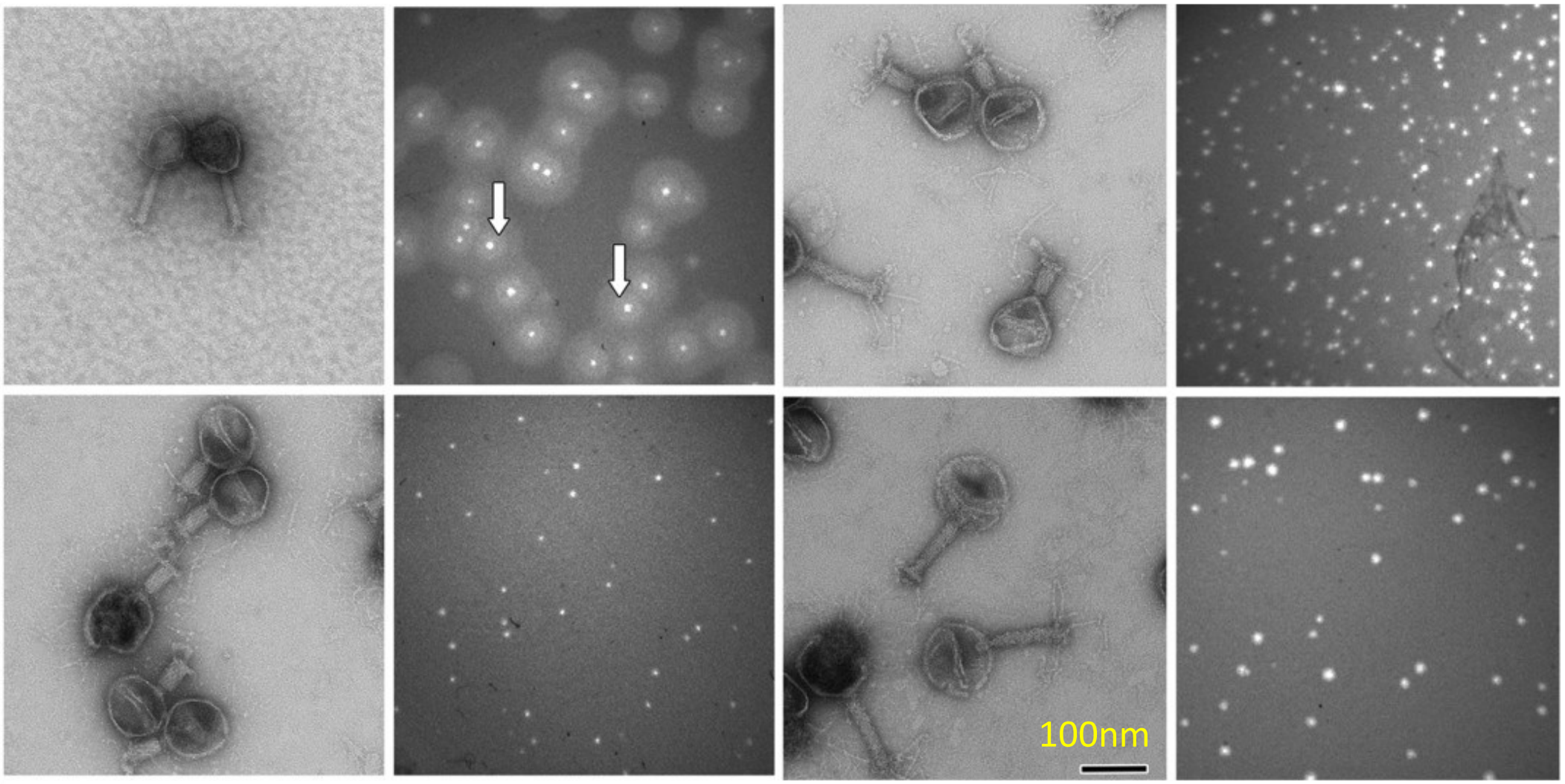
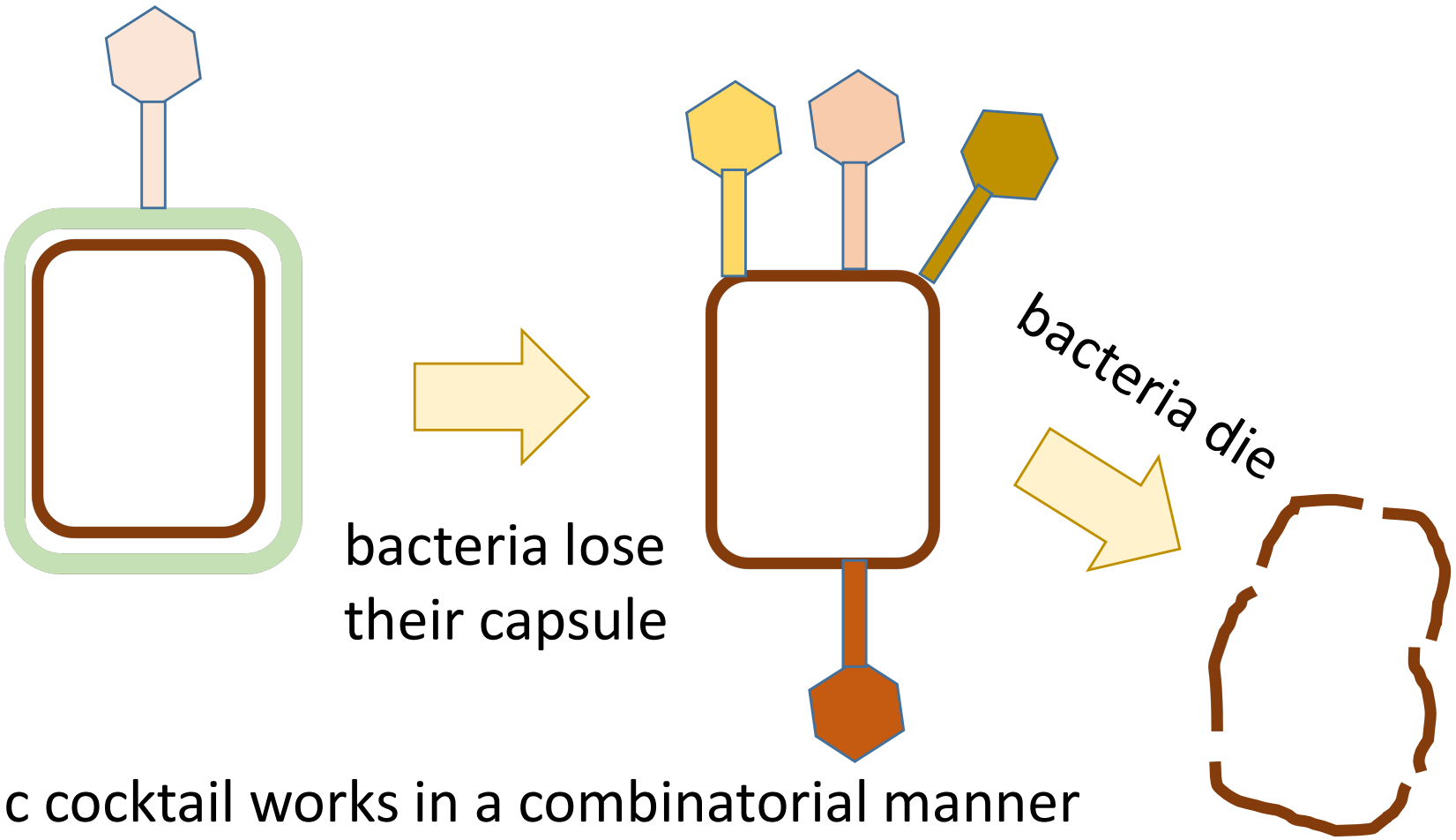


Fig. 3-53 **Glycocalyx.** Colorized micrograph of the glycocalyx *(red)* of a Gram-negative bacterium. (59,000×.)



Electron micrographs of different bacteriophages and their respective plaque morphologies



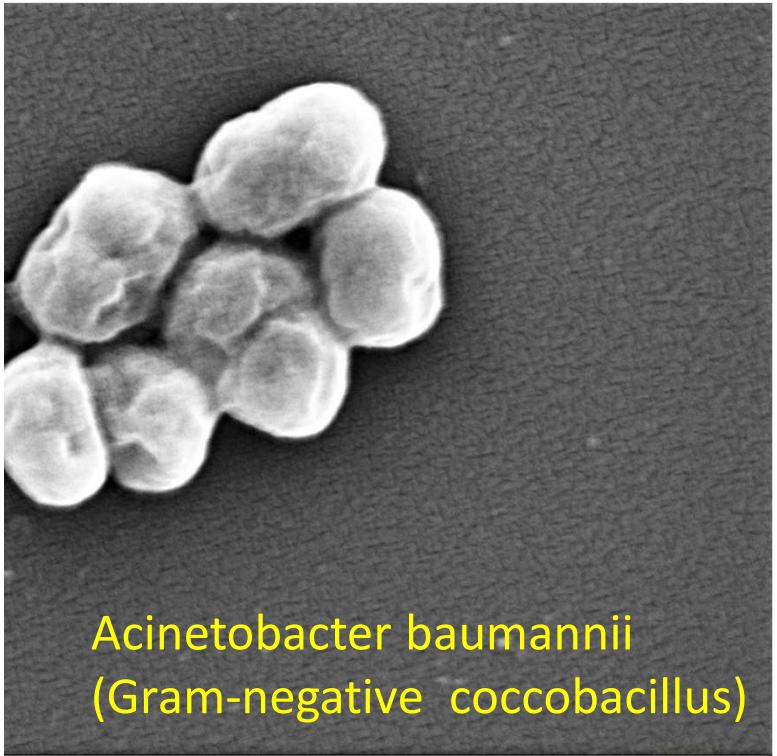
therapeutic cocktail works in a combinatorial manner

Treatment of *A. baumannii* infected wounds with the phage cocktail leads to vastly improved clinical outcomes in a mouse wound model.

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

Schooley et al. (2017) Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant Acinetobacter baumannii infection. Antimicrob Agents Chemother. 61(10): 00954-17.
Published online 2017 Sep 22. Prepublished online 2017 Aug 14. doi: 10.1128/AAC.02877-15

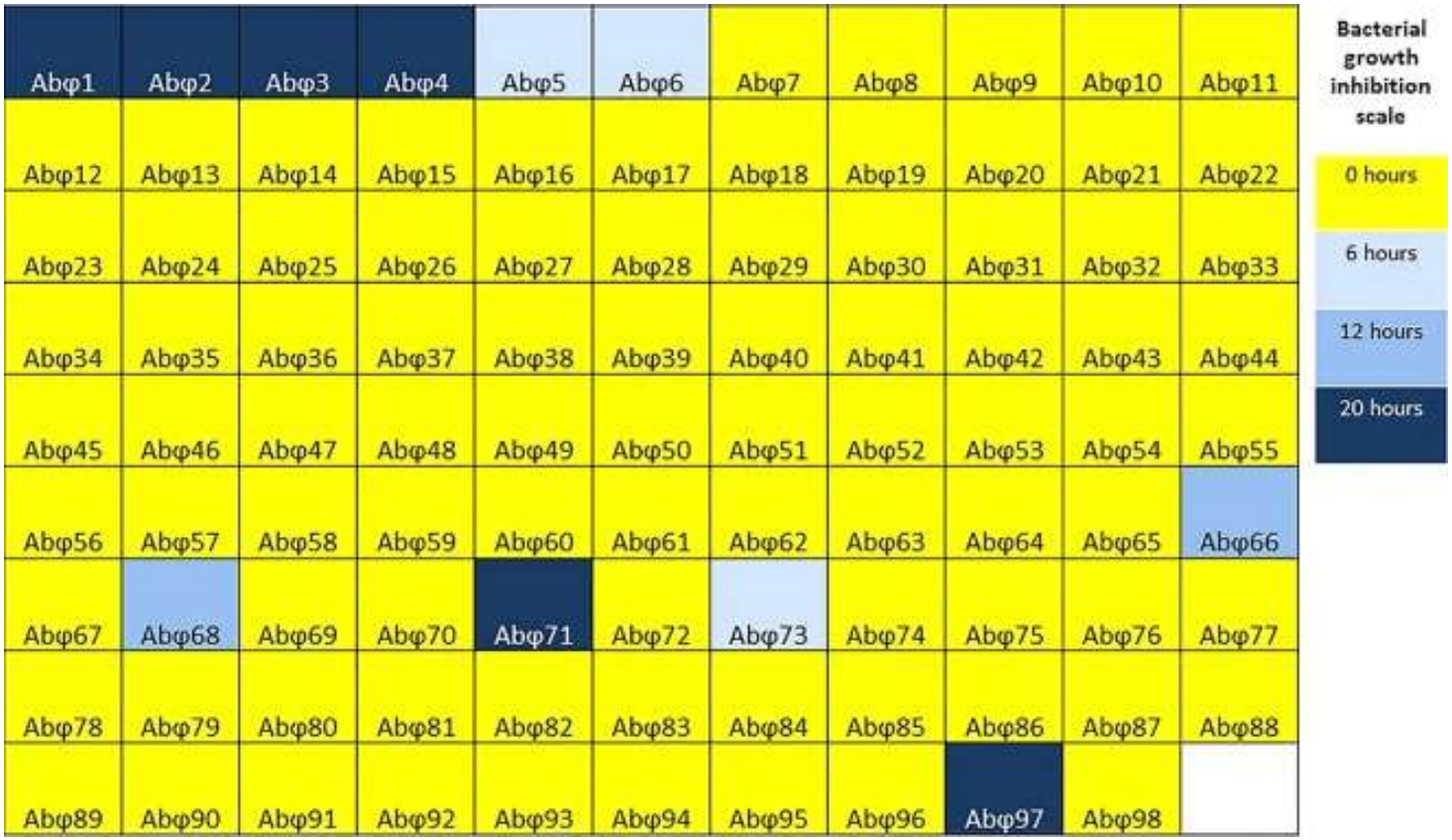
A report on a method used to produce a personalised bacteriophage-based therapeutic treatment for a 68-year-old patient with necrotizing pancreatitis complicated by an MDR (multi-drug-resistant) *A. baumannii* infection. Despite multiple antibiotic courses, the patient deteriorated over a 4-month pariod. In the absence of effective antibiotics, nine different bacteriophages with lytic activity for an *A. baumannii* isolate from the patient were isolated.



Food and Drug Administration (FDA) authorisation was obtained, to administer the phage cocktail as an emergency investigational new drug (eIND).

The outcome of the case suggests that an approach like this could be applied to similar cases, and that more efforts to investigate the use of therapeutic bacteriophages for MDR bacterial infections are warranted.

A subset of 98 phages from the Navy phage-library were individually tested against the *A. baumannii* clinical strain using the OmniLog system (Biolog, Hayward, CA). Growth of bacteria or lack of growth due to lysis from phage infection was monitored every 15 min via a redox chemical reaction employing cellular respiration as a reporter – cellular respiration from growth reduces a tetrazolium-based dye and produces a color change. If the growth is weakly positive or is negative, then respiration is slow or absent and so little to no color change is observed. The results are summarized here, where the color gradient indicates the duration of bacterial growth inhibition.

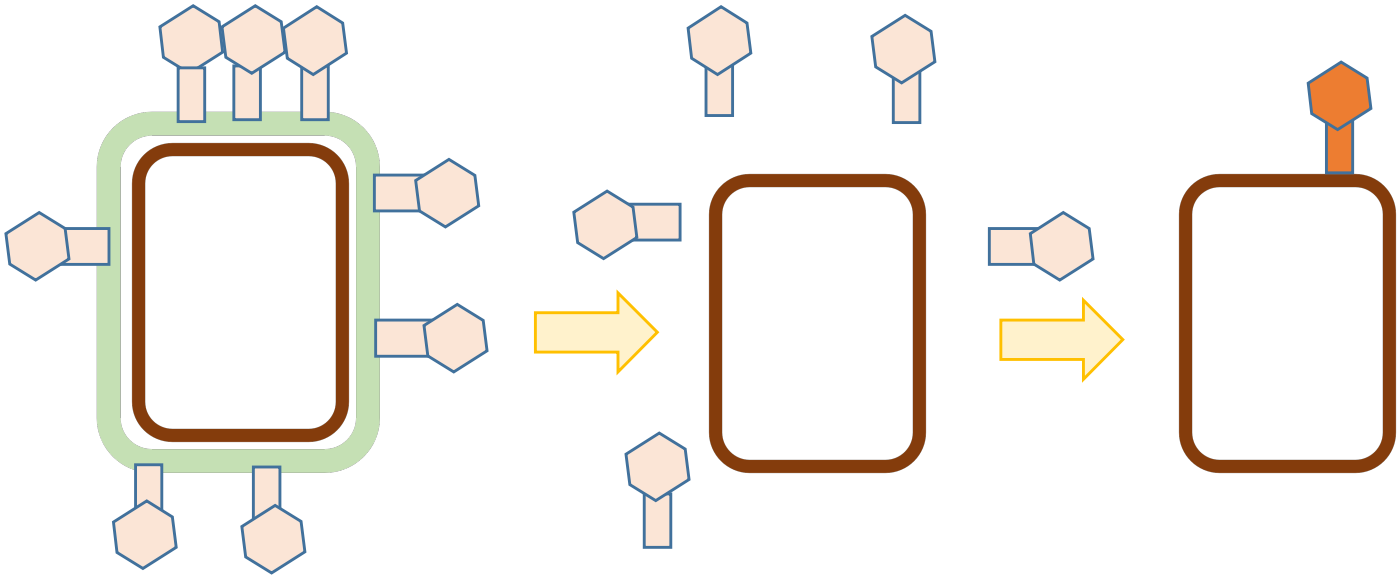


Screening of *A. baumannii* phage library against the particular clinical isolate.

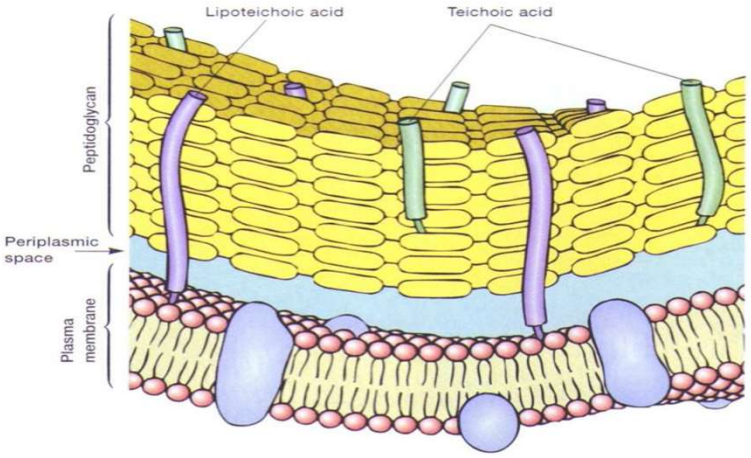
A. baumannii susceptibility to bacteriophages was determined by individually screening large panel of phages, isolated previously for their activity against A. baumannii. By targeting bacterial isolate from the patient in case, eight phages were selected to be incorporated into therapeutic cocktails.

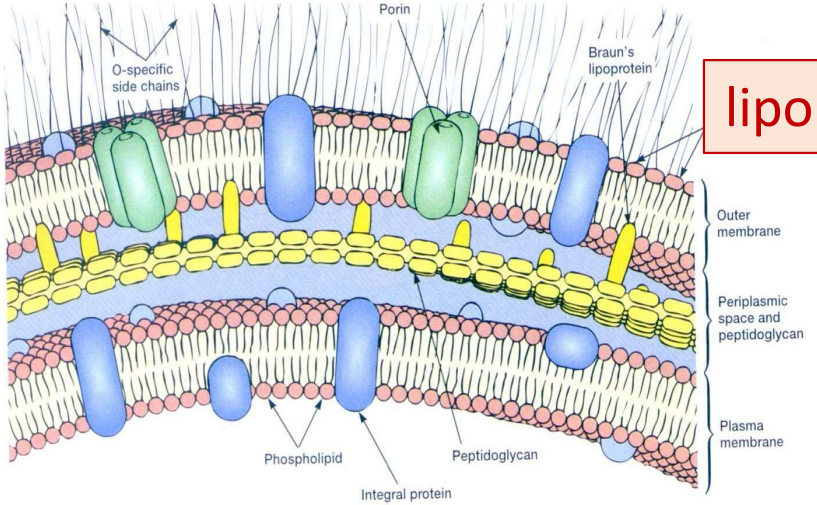
It was determined on the 8th day of bacteriophage therapy, that each of the phages had lost activity individually and in the mixture, against the new bacterial isolate that emerged in the presence of the phages. Therefore, an additional lytic phage was selected rapidly (within 72 hours) against the new isolate and combined with one of the phages from the original set. That new cocktail was then administering to the patient.

The new bacterial isolate that emerged during phage therapy had lost its capsule. Consequently it has lost specific receptors for virus attachment – concrete reason for insensitivity to viruses that were able to infect the original bacteria.



Interestingly, the phage-resistant phenotype that arose over time was associated with increased antibiotic sensitivity when phage and antibiotics were simultaneously administered.





lipopolysaccharide

An **endotoxin** from bacterial host cells (Gram-negative cell-wall) could be harmful to a patient, thus each therapeutic phage cocktail should be assessed for residual endotoxin level to be sure to meet the FDA-recommended endotoxin limitation for intravenous application.

In the end, the therapeutic treatment of the patient was successful

The intravenous bacteriophage administration was well tolerated, thus it was repeated at increasingly frequent intervals over the next 2 days.
The patient awoke from his coma and generally demonstrated on-going improvement on all fronts. Bacteriophage therapy was continued for additional 8 weeks, during which time he demonstrated further clinical improvement. Finally, he was discharged home and has subsequently returned to work.

General conclusions on phage therapy

- Personalised bacteriophage therapy is highly recommended
- Combined therapy using phage cocktail is much more efficient comparing to single phage administration
- Emergence of virus-resistant bacteria during phage therapy should be expected
- Phage therapy could sometimes recover antibiotic sensitivity – combined administration of both phages and antibiotics might be beneficial, particularly in the later phase of therapy
- Endotoxin level has to meet FDA-recommended value, particularly when phages are administered intravenously

U stvari, dva su razloga zbog koji je nužno tražiti nove, alternativne ili dopunske strategije u borbi protiv patogenih bakterija.

Odavno znamo da su probiotički proizvodi korisni za naše zdravlje. Ali nakon što je završio projekt istraživanja ljudskog mikrobioma ovo je područje postalo jedan od fokusa suvremene znanosti. Novi rezultati uvjerili su nas koliko naša mikrobiota pozitivno utječe na naše tjelesno i mentalno zdravlje. Prema tome, i da se nije razvio problem otpornosti na antibiotike, morali bismo preispitati našu primjenu antibiotika s ciljem da spriječimo uništavanje naše mikrobiote.

Uska specifičnost bakteriofaga (često ispod razine bakterijske vrste), čini ih kompliciranijima od antibiotika u personaliziranom liječenju, ali to je i izuzetno povoljna karakteristika obzirom na apsolutnu neškodljivost u odnosu na naše dobre bakterije.

OUR MICROBES HAVE SOME KEY ROLES IN OUR HEALTH

contributing significantly to our prenatal development, as well as well-being during our entire life

Do not make them collateral victims of unnecessary antibiotic treatments

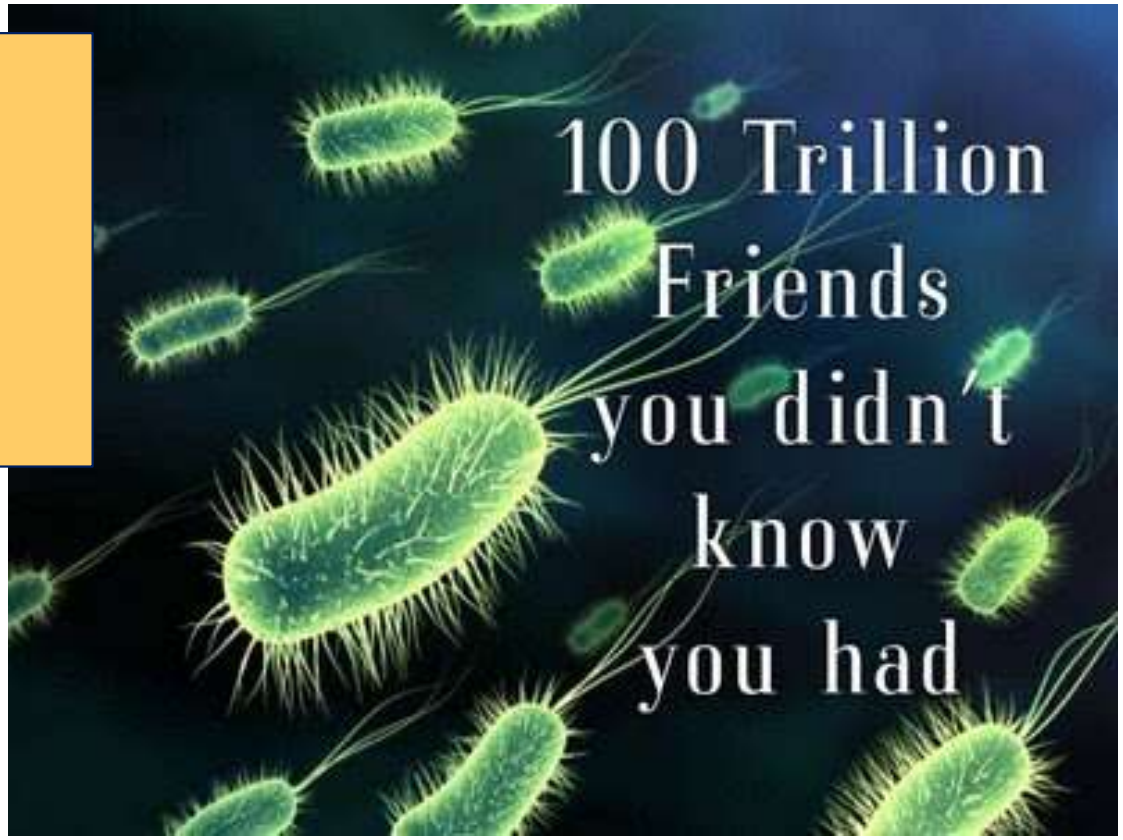
!!!

- microbial cells outnumber human cells
- prokaryotic population contributes 300-fold more protein genes

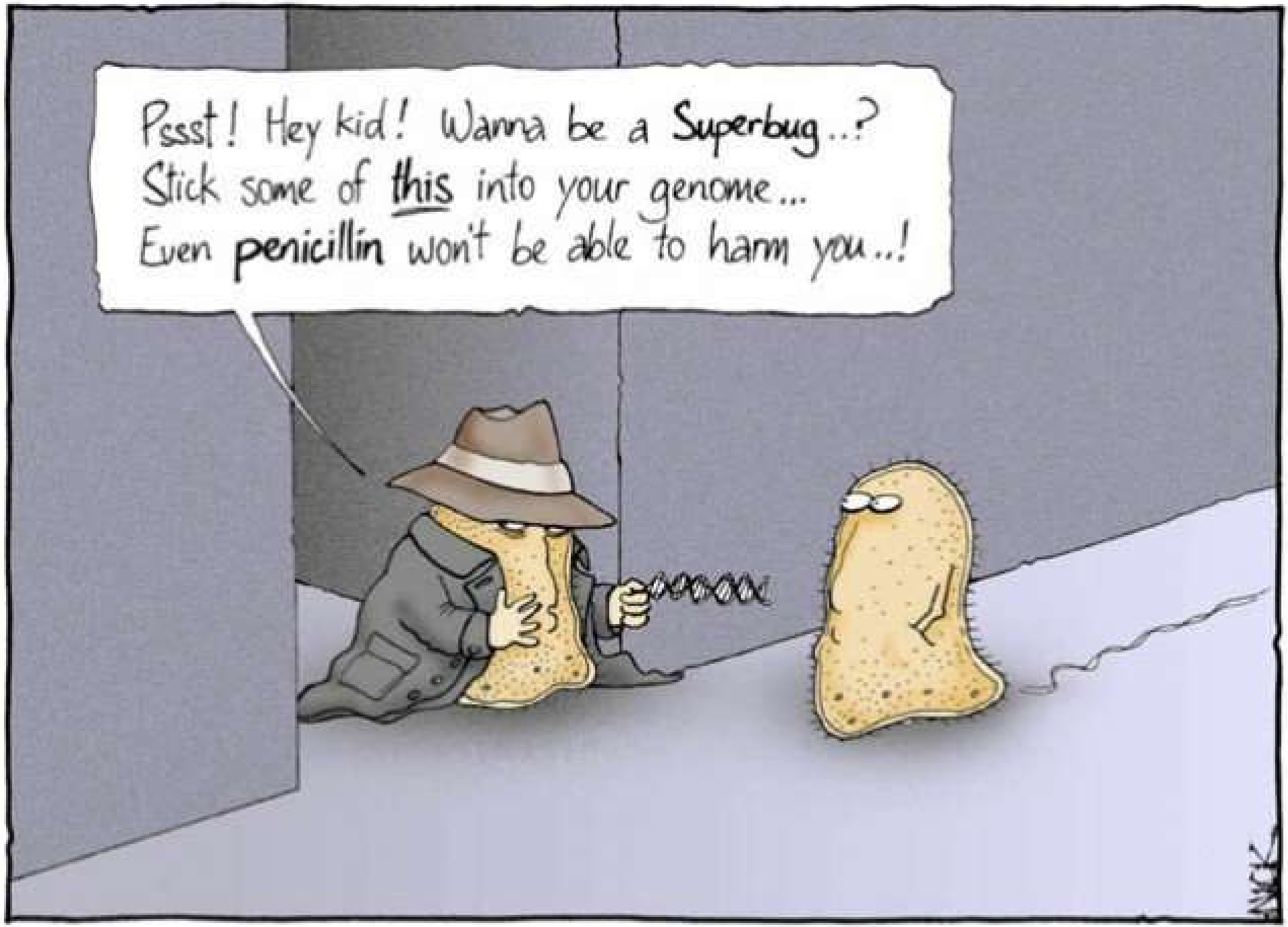
***Nature* (2012) 486, 207–214**

Projekt ljudskog mikrobioma:

- 100 trilijuna bakterija
- 95% u crijevima
- koža, usta, dišni organi i genitalije
- 2kg



Rezultati, koji su objavljeni u časopisu *Nature*, bili su impresivni. Spoznali smo da prokariotskih mikroorganizama u, odnosno na, našem tijelu ima više nego naših vlastitih eukariotskih stanica, a ukupni mikrobiom 300 puta nadmašuje ljudski genom brojem protein-kodirajućih gena.



It was on a short-cut through the hospital kitchens that Albert was first approached by a member of the Antibiotic Resistance.