Studying on pasteurellosis agent isolate from died saiga antelope

Zhumagali Koshemetov[1], Nurlan Sandybayev[1], Abylai Sansyzbay[1], Muhit Orynbayev[1], Berik Khairullin[1], Sergazi Nurabayev[1], Valentina Matveyeva[1], Marina Bogdanova[1], Gulnur Sugirbaeva[1], Madina Seisenbayeva[1], Assilbek Burabaev[2], Marlen Yessirkepov[2]

[1]Research Institute for Biological Safety Problems (RIBSP), the Republic of Kazakhstan
[2]South Kazakhstan State Pharmaceutical Academy, Shymkent, Republic of Kazakhstan
marlen-forex@inbox.ru

Abstract: The article contains the biological characterization of pasteurellosis strains isolated from dead saiga antelopes in Kazakhstan. Results demonstrated that pasteurellosis strains isolated from saiga antelopes from different periods had identical biological characteristics.
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Keywords: agar, biological characteristics, nutrient medium, pastuerellosis, saiga antelope, strain.

1. Introduction

Saiga antelopes currently inhabit the territory of Kazakhstan, Uzbekistan and migrate to Russia (Kalmykia and Astrakhanskaya oblast), Turkmenia and West Mongolia.

In 2008, the saiga antelope population was 50 000 animals. Saiga antelope belongs to the subspecies *Saiga tatarica tatarica* and inhabits Russia (North – West Caspian territories) and three oblasts in Kazakhstan (Volgo – Ural sand territories, Ustyurt and Betpak – Dala) (Mallon, 2008). In 2010 there was an epidemic of pasteurellosis and twelve thousands of these saiga antelopes died on the border between Volgogradskaya oblast and Kazakhstan (Naimark 2011, Ellis 2011, Pyatnov 2010). In 1990 in the Republic of Kalmykia (Russian Federation) a nature reserve called “Chernye Zemli” (Black Territories) was established to protect populations of saiga antelopes living in the north-western Caspian territories (Dushan, 1976).

The saiga population inhabiting western Mongolia is usually referred to as a separate subspecies called *Saiga tatarica mongolica*. This population consists of 750 animals. All other populations belong to a nominal subspecies *Saiga tatarica tatarica*. Some researchers consider Mongolian saiga as a Pleistocene subspecies and call it *Saiga borealis mongolica* (Wilson, 2005).

According to data of one organization for statistics in the Republic of Kazakhstan, in 2011 the number of saiga in the Betpakdala population was estimated at 78 thousand animals, in the Ural population there were 17, 9 thousand saiga antelopes and the Ustyurt population consisted of 6.1 thousand animals.

Numbers of saiga antelope reduce each year despite various precautions taken in Kazakhstan and in other countries (Novoselova, 2008).

Shortages of winter fodder so called “dzhute” can contribute to reducing populations; furthermore, heavy snowfall and surface ice provide very dangerous conditions for saiga antelopes. But many males die in the spring when their carcasses can be seen covering the desert, the scene resembles a battlefield. Wolf populations increase periodically and are also dangerous predators of saiga antelopes in Kazakhstan deserts. Saiga antelopes are hunted for meat and their horns are used in traditional Tibetian medicine, which is another consideration for declining populations. And, of course, the species is susceptible to infectious diseases. Before 2010 incidences of mass death of saiga from pasterelousis in Kazakhstan were recorded in 1981, 1984 and 1988.

According to data obtained by RIBSP and the Ministry of Agriculture researchers as well as mass media information saiga mass death in Kazakhstan was registered in 1981, 1984, 1988, 2010 and 2011. In the territory of former Turgai oblast in May 1981 about 100 000 of the species died and in May 1988 – about 270 000 animals died (Aikimbayev, 1985). In May 1984 more than 100 000 saigas died (Martinevskiy, 2001). In May 2010 more than 12 thousand animals died. In 1988 mass saiga death occurred in May 14 – 22, and in 2010 saiga antelopes died from 18th to 21st of May. In May 26 – 27, 2011 during the period of calving in Zhanibekskiy region in Western-Kazakhstan oblast saiga mass death happened again. 441 animals died including 364 females and 77 calves. In May 24, 2012 in Kostanaiskaya oblast 290 dead saiga antelopes were found. Early in May 19, 2012 the veterinary service found 623 dead saiga antelopes (Lundervold, 2004).

An international acclaimed expert Richard Kock (Royal Veterinary College, London) and a national expert Adylkasym Zhakypbayev (veterinary, virologist, Kazakhstan) were invited to conduct a more detailed study on the cause of mass death of saiga and to make recommendations for prophylaxis. This research was funded by international non-governmental organizations (Flora & Fauna International, UNDP, ACBK).

According to Richard Kock from the Royal Veterinary College in London, saiga mass death in Western Kazakhstan in 2010 – 2011 was caused by overeating.

The expert explained that spring 2010 – 2011 was an atypically hot and humid season, which caused the grass on the territory to become especially rich. According to his research, overeating watery grass brought abnormalities in gastrointestinal tracts of saiga antelopes. These abnormalities then induced production of surplus gas, which affected the animals' lungs and caused death from asphyxia.

However, the expert admits that there may have been secondary causes contributing to mass death such as infection by Pasteurellae bacteria (Kock, 2012).

RIBSP researchers isolated *Pasteurella* during laboratory examination of biological material taken from dead saiga in Turgaiskaya oblast in 1981 and from Western Kazakhstan in 2011. On the basis of the study implemented by RIBSP researchers it was determined that one of the main reasons of saiga mass death in Kazakhstan was *Pasteurella* (Koshemetov Z, 2014, Burabaev A, 2013).

An inter-departmental commission consisting of representatives from veterinary, medical, zoological and environmental organizations concluded that "saiga mass death was caused by a Pasteurellosis outbreak" (Grachev, 2010).

The basic source of this infection is from sick animals as well as from clinically healthy animals that have been in contact with sick animals. Carriage of *Pasteurellae* plays significant role in pateurellosis epizootology. In some unfavorable farms it reaches 70% among cattle, 50 % among sheep, 45 % - among pigs, more than 50 % among rabbits and 35-50 % among chickens (Sidorchuk, 2007).

In this connection biological characterization of pasteurella strains isolated from dead saiga antelopes in Kazakhstan is very important for further detailed studies of isolated strains and to prepare diagnoses and means for prophylactic action.

This article contains basic results of determination of isolated pasteurella strains and serovar as well as biological characteristics.

MATERIAL AND METHODS

Agent.

Epizootic strains "Saigachiy" isolated from dead saiga in Turgaiskaya oblast on August 17, 1988 and Pasteurella/Saigak 1/2011/ZKO/KZ/ isolated in West-Kazakhstan oblast in 2011 were used in this work.

Methods of media preparation

Media (agar and broth) produced by Himedia and Gissa medium with lactose, Endo-FPH (fish protein hydrolysate), Kligler - FPH, Simmons - M, Kligler with glucose, rhamnose media were prepared according to the manufacturer's instructions.

Pasteurella cultivation

A bacteriologic loop or spreading rod was used for bacteriologic inoculation in solid media. Lines were traced by the bacteriologic loop on the surface of a solid medium and thus bacterium cells inoculated the medium. After inoculation, plates were closed and bottoms were turned upwards. The legend was written on the bottom of plates and on the upper parts of test tubes.

For inoculation in a liquid medium the bacteriologic loop was immersed into the medium and the material was spread on the walls of the tube after the material was washed with the medium.

After thorough bacteriologic inoculation, bacteria were cultivated at 37 ^{o}C for 24 – 48 hours.

Gramm staining of smears

Cristal violet was poured on the fixed smear. After 2 minutes of exposition it was washed with water. 96 % ethanol was used to decolorize the smear for 30 seconds and then it was washed with water again.

Pfeiffer fuchsine was used to finish staining for 1 minute; the smear was then washed with water and dried.

Mobility test

Samples were studied for mobility after 24 hours of incubation at 37 °C. The colonies were covered by a cover glass covered by immersion oil and the colonies were examined in the light of the microscope. Pasteurellosis bacteria are immobile.

Haemolytic activity

This test was conducted in blood agar (peptone agar with 5 % defibrinated sheep blood), which was inoculated with broth cultures of the studied samples. All cultures were incubated at (37±1) °C. And the width of the haemolysis zone was examined daily (in 24, 48 hours). The haemolysis zone was a clearly bordered totally transparent zone due to complete destruction of erythrocytes. The pateurellosis agent does not cause haemolysis of sheep erythrocytes.

Indole test

Indole was detected using a test paper. The test paper was made from 10- 12 sm long pieces of filter paper saturated with hot oxalic acid and dried in a

thermostat. The test paper was then placed vertically into the tube containing bacteria cultivated in meat-pepton broth. The lower end of the test paper was not allowed to touch the medium. The test tube was incubated at 37 °C for 24 – 72 hours. The lower part of the test paper becomes pink when indole is released.

Urease determination

For urease determination 2 -3 drops of 24-hours broth culture were placed into the tube-containing medium with carbamide. It was then cultivated for 20 – 24 hours at 37 ºC. If urease ferment was present the medium turned red. Pasteurellosis agent does not cause medium reddening.

Biological research

Biological research was done to determine pathogenic characteristics of pasteurella cultures and if necessary to detect an agent in the pathologic material.

Pathogenicity of pasteurella cultures was determined on white mice weighing 16 – 18 gr 0,2 sm3 of 18 – 24 – hours' broth culture was injected subcutaneously to 2 white mice. *P. multocida* virulent strains of serovar B that cause haemmorhagic septicemia kill infected laboratory mice within 24 – 72 hours post injection. Low virulent strains of serovars A and D cause pneumonia and kill laboratory mice within 7 days. *P.haemolytia* causes death of laboratory mice only after intra abdominal injection. Other types of *Pasteurella* are nonpathogenic for laboratory animals.

For adaptation of pasteurella bacteria in agar (Himedia) with 10 % bovine sera, it was inoculated and incubated for 24 – 48 hours at 37 °C.

"Saigachiy" and Pasteurella/Saigak/2011/ZKO/KZ strains were used for these tests. Serially 10 inoculations (passages) were conducted for adaptation of "Saigachiy" and Pasteurella/Saigak/2011/ZKO/KZ strains in media produced by Himedia (nutrient agar and broth). Morphology of the colonies was studied at the end of each passage (figures 1 - 4), virulence in white and Gramm staining of smears (figures 5 - 6). The strain colonies were evaluated visually.

Fig 1. "Saigachiy"strain colonies in agar

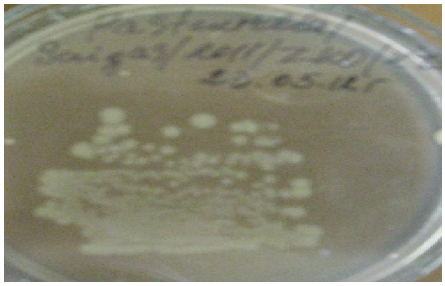

Fig 2. Pasteurella/Saigak/2011/ZKO/KZ strain colonies in agar

Fig 3. Growth of "Saigachiy" isolate in nutrient broth

Fig 4. Growth of Pasteurella/Saigak/2011/ ZKO/KZ strain in nutrient broth

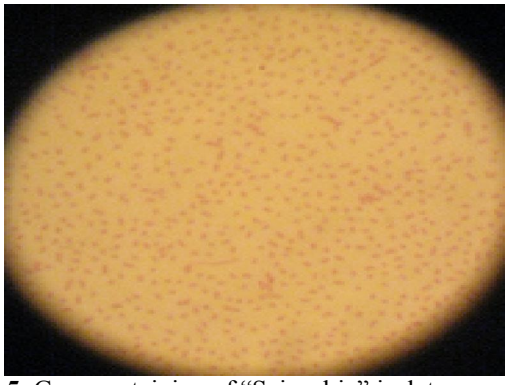

Fig 5. Gramm staining of "Saigachiy" isolate

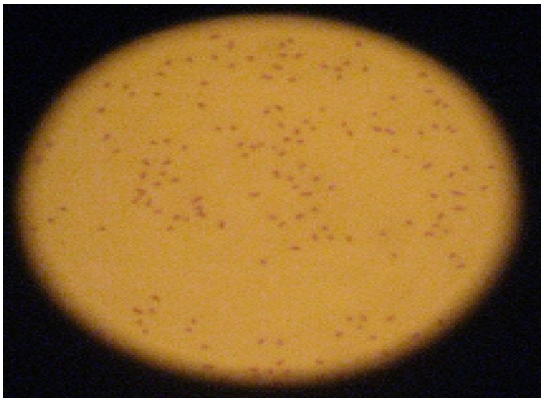

Fig 6. Gramm staining of Pasteurella/Saigak/ 2011/ ZKO/KZ strain

In 24 hours after inoculation in nutrient broth both strains formed even colonies.

The growth of pasteurella strains in liquid media was accompanied by weak opacity. In 36 hours the medium cleared and sediment formed on the bottom of the tube. When shaking the tube the sediment aroused as braid.

When staining pasteurella bacteria in smears looked like Gramm-negative ovoids or coccus bacteria situated singlewise or in pairs.

Pathogenicity of bacterium cultures was determined in white mice weighing 16-18 gr. Cultures of the studied strains caused death of infected mice in 12 hours and 35 minutes ("Saigachiy") and 12 hours and 5 minutes (Pasteurella/Saigak/2011/ZKO/KZ). Dotted hemorrhage was observed in lungs, liver, spleen and kidneys of the dissected mice.

During the course of the experiment pasteurella strains "Saigachiy" and Pasteurella/Saigak/2011/ZKO/KZ were replenished and adapted in nutrient agar and nutrient broth with 10 % bovine serum. These isolates were cultivated at 37 °C for 20 – 48 hours.

It was established that after 10 serial passages of the bacteria in nutrient agar and broth "Saigachiy" and Pasteurella/Saigak/2011/ZKO/KZ strains did not change their initial biological characteristics.

It is known that there are six types of pasteurella bacterium: *P.multocida*, *P.haemolytica*, *P.ureae*, *P.pneumotropica*, *P.aerogenes*, *P.gallinarum*. These types cause differently localized and courses of infectious diseases of domestic and wild animals, birds and human beings. Leading etiological significance in infectious pathology of animals and birds belongs to *P. multocida* and *P.haemolytica*.

P. multocida causes hemorrhagic septicemia in animals, birds cholera and lungs pasteurellosis complicating respiratory infections of viral and mycoplasma etiology.

P. haemolytica causes pneumonia in cattle and sheep of all ages as well as septicemia in new-born sheep.

Experiments were done to differentiate strains of "Saigachiy" and Pasteurella/Saigak/2011/ZKO/KZ and to determine type and serovar (Table 1).

Table 1. Basic differentiating characteristics of pasteurella types

Characteristics	*P.multo-cida*	*P.pneumo-tropica*	*P.haemo-lytica*	*P.ureae*	*P.aero-genes*	*P.gallinarum*
Mobility	-	-	-	-	-	-
Haemolysis in blood agar	-	-	+	-	-	-
Indole formation	+	+	-	-	-	-
Presence of urease	-	+	-	+	+	-
Biological test in white mice, death days	7 days	-	-	-	-	-
Notes :1 – «+» - positive result ; 2 – «-» - negative result						

The results of mobility tests of pasteurella "Saigachiy" and "Pasteurella/Saigak/2011/ZKO/KZ" strains are presented in figures 7 and 8.

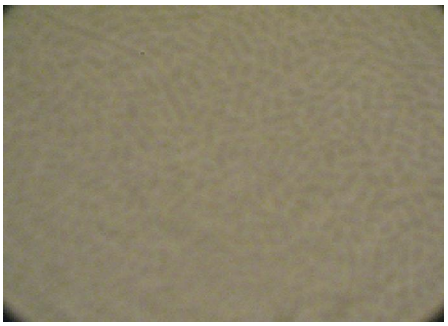

Fig 7. Mobility test of "Saigachiy" strain

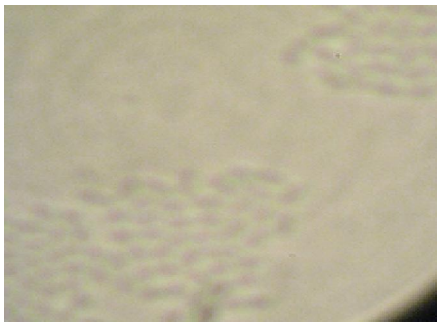

Fig 8. Mobility test of "Pasteurella/Saigak/ 2011/ ZKO/KZ" strain

The results showed that both strains were immobile, which is characteristic of *Pasteurella*.

Hemolytic activity results of both strains are presented in figure 9.

Fig 9. Hemolysis test

The studied samples did not cause haemolysis of sheep erythrocytes in 48 hours of incubation at 37 °C.

Indole formation in the studied strains was revealed using test paper. The results are presented in figures 10 and 11.

Fig 10. Indole test of "Saigachiy" strain

Fig 11. Indole test of Pasteurella/Saigak/2011/ ZKO/KZ

The pictures above show that the lower part of the test paper became pink after incubation for 72 hours at 37 °C confirming presence of pasteurella bacterium.

Urease determination in the studied samples is presented in pictures 12 and 13.

Fig 12. Urease determination test of "Saigachiy"

Fig 13. Urease determination test of Pasteurella/Saigak/2011/ZKO/KZ

As demonstrated in these pictures the medium was not red after introduction of 24-hours incubated broth culture. This is characteristic for *Pasteurella*.

Biological tests were done on white mice.

Mice died 12 and 13 hours after infection with the studied samples. On the basis of the conducted research it can be concluded that the strains "Saigachiy" and Pasteurella/Saigak/2011/ZKO/KZ belonged to *P. multocida*, serovar B.

The differential Table 2 was made on the basis of conducted experiments.

Table 2. Differentiation results of the studied samples of *Pasteurella*

Characteristics	"Saigachiy"	Pasteurella/Saigak/2011/ ZKO/KZ
Mobility	-	-
Hemolysis in blood agar	-	-
Indole formation	+	+
Presence of urease	-	-
Biotest in mice, death in….	12 h 35 min	12 h 06 min
Notes: – «+» - positive result; 2 – «-» - negative result		

Biochemical characteristics of the strains "Saigachiy" and Pasteurella/Saigak/2011/ZKO/KZ were also studied. Studying the enzymatic characteristics of isolated pasteurella cultures is one the methods used for its identification. Enzymatic characteristics of "Saigachiy" and Pasteurella/Saigak/2011/ZKO/KZ carbohydrates were studied in the following media containing different concentrations of carbohydrates: Gissa with lactose, Endo-FPH, Kligler – FPH, Simmons – M, Kligler with glucose, rhamnose. Results are presented in figures 14 – 19.

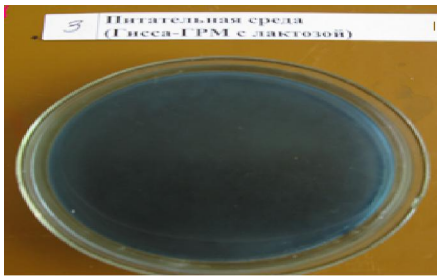

Fig 14. Control medium Gissa – FPH with lactose

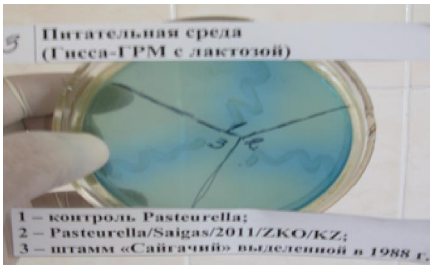

Fig 15. Gissa - FPH with lactose inoculated with "Pasteurella /Saigas/2011/ZKO/KZ" and "Saigachiy"

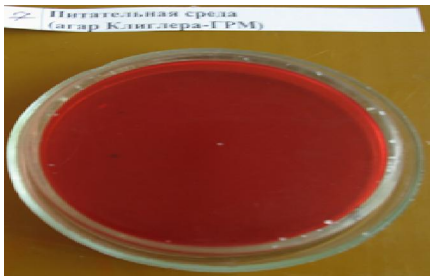

Fig 16. Control Kligler agar - FPH

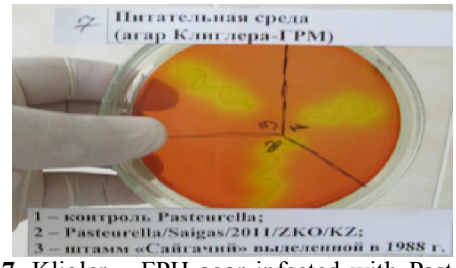

Fig 17. Kligler – FPH agar infected with Pasteurella /Saigas/2011/ZKO/KZ and "Saigachiy" strains

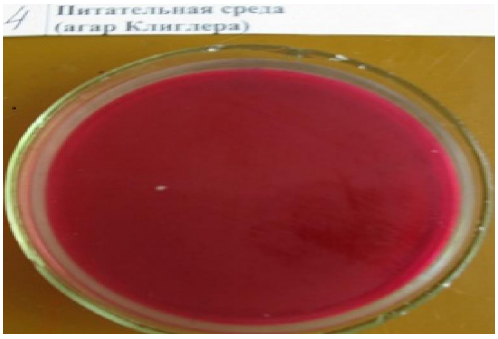

Fig 18. Control Kligler agar

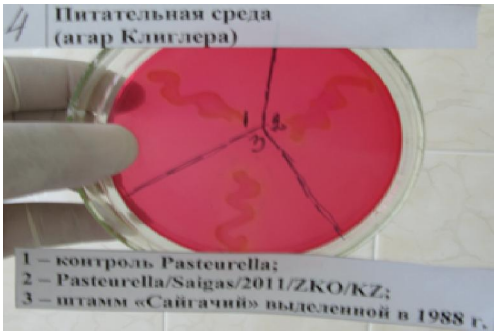

Fig 19. Kilgler agar inoculated with Pasteurella/Saigas/2011/ZKO/KZ and "Saigachiy" strains

When the strains Pasteurella/Saigas/2011/ZKO/KZ and "Saigachiy" are inoculated into Gissa – FPH medium, carbohydrate fermentation starts and the medium becomes blue.

Yellow staining i.e. lactose fermentation is clearly seen around the colonies formed after inoculation of the strains into the Kligler – FPH agar.

According to data from the experiment pasteurella strains caused weak fermentation of carbohydrates. The studied pasteurella strains did not cause carbohydrate fermentation or did not colonies grow at all in other media.

Aggregate results of the growth of isolated strains in various media are presented in figure 20.

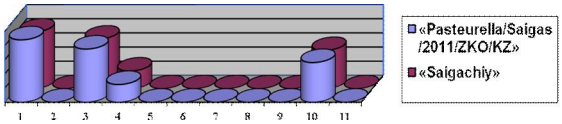

Fig 20. Results of the isolated strains growth in various media. 1 – Gissa – FPH with lactose; 2 – Endo – FPH; 3 – Kligler – FPH; 4 – Kligler; 5 – Simmons – M; 6 – agar with glucose; 7 – agar with rhamnose; 8 – agar with mannose; 9 – polymixine medium; 10 – FPH – agar; 11-#14 FPH (Simmons cytrate agar).

Antibiotics such as neomycin, carbenicillin, bicillin -5, bicillin – 3, tetracycline, cephazoline, ceftriaxson, streptomycin, ampicilline, gentamycin, lincomycin, benzilpenicillin sodium salt were used to study the susceptibility of pasteurella strains to these antibiotics. Different concentrations of antibiotics were tested in this experiment. Results of the tests for susceptibility to antibiotics are presented in Table 3.

Table 3. Antibiotic susceptibility testing results

Antibiotic susceptibility level	Pasteurella strains	
	"Saigachiy"	"Pasteurella/Saigas/2011/ZKO/KZ"
Gentamycin, mg/sm^3 (produced in Germany)	0,5	0,5
Benzilpenicillin, unit/sm^3 (produced in Germany)	10	10
Lincomycin, mg/sm^3 (produced in Shymkent)	0,3	0,3
Ampicilline, mg/sm^3 (produced in Germany)	0,01	0,01
Ceftriaxon, mg/sm^3 (produced in Germany)	0,0001	1
Cephazoline, mg/sm^3 (produced in Germany)	1	1
Streptomycin, mg/sm^3 (produced in Shymkent)	10	10
Streptomycin, mg/sm^3 (produced in Germany)	10	10
Bicillin – 3, unit/sm^3 (produced in Shymkent)	600	600
Bicillin – 5, unit/sm^3 (produced in Shymkent)	120	120
Carbenicillin, mg/sm^3 (produced in Germany)	0,1	1
Neomycin, mg/sm^3 (produced in Germany)	1	1
Penicillin unit/sm^3, streptomycin mg/sm^3 and amphotericine B µg/sm^3 (produced in Germany)	100 – 0,1 – 0,25 accordingly	100 – 0,1 – 0,25 accordingly
Tetracyclin, mg/sm^3 (produced in Germany)	1	0,1

Microorganisms are usually divided according to the following categories; susceptible, moderately susceptible and resistant to antibiotics. The strains tested in this study were categorized as susceptible microorganisms. Bacterial growth of "Saigachiy" and "Pasteurella/Saigas/2011/ZKO/KZ" colonies was suppressed at minimal concentrations of the above-mentioned antibiotics. 5 % erythrocytes of cock, sheep, goat, donkey, turkey, cattle, goose, rabbit and horse were used in the experiment of erythrocytes haemolysis.

Nutrient agars containing 10 % bovine serum and 5 % erythrocytes of the above mentioned animals and birds were inoculated with "Saigachiy" and Pasteurella/Saigas/2011/ZKO/KZ strains and incubated for 48 hours at 37 °C.

"Saigachiy" and Pasteurella/Saigas/2011/ZKO/KZ strains do not cause haemolysis of erythrocytes of different animals and birds and this is characteristic for *Pasteurella*.

We used media with different pH levels (5,0, 6,24, 7,4, 8,5) to study the effect of pH on the growth of pasteurella strains. Agar was prepared with 10% bovine serum and then pH was adjusted to the necessary level. After that, "Saigchiy" and "Pasteurella/Saigas/2011/ZKO/KZ" strains were inoculated into those media. Three passages were conducted in order to obtain more reliable results and at the end of the experiment biological tests were done on the white mice.

The results showed that 6,26 – 8,5 pH did not influence the virulence in either strain. The "Saigachiy" strain caused death of white mice in 12 hours 30 minutes – 15 hours 30 minutes post infection. The same results were obtained for the Pasteurella/Saigas/2011/ZKO/KZ strain. And both tested strains did not grow in nutrient agar having a pH level of 5,0.

Also, to study the influence of bovine serum concentration in agar on the pasteurella strains growth three passages of both strains were tested in each of the following nutrient agars with different concentrations of bovine serum (1. without serum, pH 7,4; 2. 5 % serum pH 7,4; 3. 10 % serum pH 7,4; 20 % serum pH 7,4). Biological tests were done.

According to these results, bovine serum concentration in the nutrient agar did not influence the virulence of pasteurella strains. The laboratory mice died in 12 h 06 min – 19 h 03 min post infection irrespective of bovine serum concentration.

To study the influence of different animals' sera on virulence and growth of the pasteurella strains the following additions were made in to the nutrient media; 10% bovine serum, sera of goat, sheep, donkey and horse. After preparation of media pasteurella strains "Saigachiy" and Pasteurella/Saigas/2011/ZKO/KZ were inoculated into these media with different sera. At the end of the experiment biological tests were done on the obtained materials.

The studied pasteurella strains grew perfectly in nutrient agars containing 10 % bovine serum, sera of goat, sheep, donkey and horse. After three passages they remained virulent and caused death of laboratory mice in 12 – 13 hours post infection.

CONCLUSION

During these experiments nutrient agar was replenished and adapted and broth pasteurella strains "Saigachiy" isolated in 1988 and "Pasteurella/Saigas/2011/ZKO/KZ" isolated in 2011. Ten passages were conducted in each medium at 37

°C for 18 – 24 hours. Biological testing showed that laboratory mice infected with "Saigachiy" and "Pasteurella/Saigas/2011/ZKO/KZ" died within 12 – 13 hours.

These results establish that both studied strains isolated from saigas during epizooty outbreaks are biologically identical and belong to serovar B of *P. multocida*.

Pasteurella strains "Saigachiy" and "Pasteurella/Saigas/2011/ZKO/KZ" did not grow in the following media: Endo – FPH, Simmons – M, agar with glucose, agar with rhamnose, agar with mannose, polymixine medium and # 14 FPH (Simmons cytrate agar). But they did grow well in FPH – agar. The studied samples fermented lactose in nutrient media Gissa – FPH with lactose, Kilgler – FPH and Kligler.

The results show that 6,26 – 8,5 pH of a medium did not influence the virulence and growth of pasteurella strains. "Saigachiy" strain causes death of white mice within 12 h 30 min – 15 h 30 min post infection irrespective of the pH level of the medium. The same results were obtained for the "Pasteurella/Saigas/2011/ZKO/KZ" strain.

Corresponding Author:
Dr. M. Yessirkepov,
South Kazakhstan State Pharmaceutical Academy,
160000 Republic of Kazakhstan, Shymkent, Al-Farabi sq., 1
E-mail: marlen-forex@inbox.ru

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