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CONTENTS

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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
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VIROLOGICAL SURVEILLANCE OF ACUTE FLACCID PARALYSES IN BULGARIA IN THE PERIOD 2001-2006

N. Korsun, Z. Mladenova, Sn. Gyurova

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SUMMARY

After the certification of Europe as polio-free region the risk of wild poliovirus importation from polio-endemic countries as well as the risk of appearance of vaccine-derived poliovirus continues to exist. In connection with this the virological surveillance of cases of acute flaccid paralyses in children aged under 15 years is very important. The results of virological investigations of patients with suspected poliovirus etiology of diseases conducted in the National Reference Enterovirus Laboratory during 2001-2006 are presented. In this article the performance of main indicators of quality of AFP surveillance in Bulgaria is also shown.

Key words: poliomyelitis, poliovirus, acute flaccid paralysis

INTRODUCTION

In 1988 the World Health Assembly of the World Health Organization (WHO) resolved to eradicate poliomyelitis globally. Since then substantial progress has been made toward that goal. The number of polio cases has reduced from over 350 000 in 1988 to 1979 in 2005, representing a greater than 99% reduction. The number of polio endemic countries has decreased from more than 125 in 1988 to just four at the beginning of 2006 (Nigeria, India, Pakistan, Afganistan). Three from the 6 WHO regions were certified as free from indigenous wild poliovirus - the Region of the Americas (1994), the Western Pacific Region (2000) and the European Region (2002). No cases caused by indigenous PV2 have been reported in the world since September 1999 (6).

Four main strategies are used in the Polio Eradication Initiative: achieving and maintaining high routine immunization coverage; performing of supplementary immunization activities and mopping-up campaigns; acute flaccid paralysis (AFP) surveillance (1).

Effective surveillance of AFP in children aged under 15 years is considered one of the key element of the initiative to achieve and confirm global poliomyelitis eradication. This surveillance system guarantees rapid detection of virulent polioviruses and the implementation of opportune antiepidemic measures.

The quality of AFP surveillance is monitored using standard indicators to measure the sensitivity of AFP reporting, the completeness and timeliness of specimen collection, and the quality and proficiency of laboratory support.

The certification standard surveillance is estimated by two main indicators: the ability to detect at least one case of non-polio AFP for every 100 000 children aged under 15 years; two adequate specimens collected from at least 80% of cases of AFP; all polio stool specimens should be processed in a laboratory accredited by WHO. Adequate specimens mean two stool specimens collected 24-48 hours apart within 14 days of paralysis onset and received in the laboratory in acceptable condition (1).

WHO established a global network of virology laboratories to support the Global Polio Eradication Initiative. The network analyses stool samples from people with cases of AFP for the presence of polioviruses. The polio laboratory network comprises 123 national laboratories, 15 regional reference laboratories and 7 global specialized reference laboratories (6). The National Reference Enterovirus Laboratory, Sofia is a part of global polio laboratory network. It is only laboratory in Bulgaria accredited by WHO and performing virological investigations of AFP cases in children aged under 15 years.

MATERIALS AND METHODS

Specimen collection. Two stool specimens were collected 24 hours apart from children aged under 15 years with AFP or paresis n.facialis.

Virus isolation and typing. The stool specimens were processed according to standard protocols for virus isolation and characterization (2). Virus isolation was performed on RD and L20B cell cultures. Viral isolates were identified by microneutralization test according to WHO' recommended standard methods. Type-specific poliovirus antisera were provided by National Institute of Public Health and the Environment (RIVM, Bilthoven, the Netherlands).

Intratypic differentiation was performed at the RRL in Italy by ELISA using cross-adsorbed type specific polyclonal antibodies (RIVM) and PCR amplification with specific primers (CDC) according to the WHO guidelines.

RESULTS AND DISCUSSION

On Figure 1 is presented the accomplishment of main indicators of the quality of AFP surveillance in Bulgaria. The figure clearly demonstrates performance >90% of all WHO requirements regarding AFP control. Hence the sensitivity of AFP surveillance was maintained at certification-standard levels in the period 2000-2006 (4).

With goal to do not omit circulation of wild or genetically changed vaccine polioviruses National Enterovirus Laboratory, Sofia investigates virologically all cases of AFP in children aged under 15 years: cases of Guillain-Barre syndrome and other polyradiculoneuritis, transversale myelitis, postvaccinal paresis and other. The virological study of neuritis n.facialis cases also have goal following up of circulation of poliomyelitis and nonpoliomyelitis enteroviruses.

The results of the virological investigations of patients with suspected poliovirus etiology of diseases in the National Enterovirus Laboratory, Sofia during the period 2001-2006 are presented in table 1. A small poliomyelitis outbreak occurred from March to May 2001 in Bulgaria against the background of 10 years epidemiological prosperity. Three cases of paralytic poliomyelitis were registered in unvaccinated Roma children aged 13, 26 and 3 months in Bourgas and Yambol. Wild poliovirus type 1 was isolated from two ill and two asymptomatic Roma children. Its North Indian origin was proved by genetic analysis of the wild polio isolates carried out in RRL-Rome and CDC-Atlanta (5).

Since the poliomyelitis outbreak in 2001 wild poliovirus, as well as vaccine-derivate poliovirus (VDPV) have been not

ABBREVIATIONS USED IN THIS PAPER:

Regional Reference Laboratory-RRL, Enzime-linked immunoassay-ELISA, Cross-absorbed type specific polyclonal antibodies-RIVM, Polymerase Chain reaction-PCR

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TABLE 1
RESULTS OF VIROLOGICAL INVESTIGATIONS OF CHILDREN UNDER 15 YEARS OF AGE WITH ACUTE FLACCID PARALYSES AND PARESIS N. FACIALIS IN BULGARIA DURING THE PERIOD 2001-2006

CLINICAL DIAGNOSIS		2001	2002	2003	2004	2005	2006 (until Nov 15)
ACUTE FLACCID PARALYSES	No. of cases/ isolates (%) Serotype of isolates	43 / 7 (16,3%) 2- PV 1 wild 1 - PV 1 SL 2 - PV 2 SL 1 -PV 3 SL 1 - ECHO 30	36 / 5 (13,9%) 1 - PV 1 SL 1 - PV 2 SL 1 - ECHO 1 2 - ECHO 11	37 / 7 (18,9%) 2 - PV 1 SL 3 - PV 2 SL 1 - ECHO 7 1 - ECHO 25	18 / 4 (22,2%) 1 - PV 3 SL 1 - ECHO 6 1 - ECHO 7 1 - ECHO 30	32 / 3 (9,38%) 1 PV 1 SL 1 mixture PV 2 + PV 3 SL	21 / 3 (14,29%) 3 PV 3 SL
PARESIS N.FACIALIS	No. of cases/ isolates (%) Serotype of isolates	45 / 1 (2,2%) 1 - Cox B	114 / 4 (3,5%) 1 - ECHO 13 1 - ECHO 14 1 - ECHO 15 1 - ECHO 24	157 / 6 (3,8%) 1 - Cox B 1 - ECHO 6 2 - ECHO 13 1 - ECHO 14 1 - ECHO 25	119 / 6 (5,04%) 2 - Cox B 2 - ECHO 6 1 - ECHO 7 1 - ECHO 12	189 / 5 (2,65%) 1 - PV 2 SL 1 -ECHO 4 1 -ECHO 11 1 -ECHO 21 1 -ECHO 25	154 / 8 (5,19%) 1 PV 3 SL 1 Cox A9 4 - ECHO 13 1 - ECHO 14 1 - ECHO 20

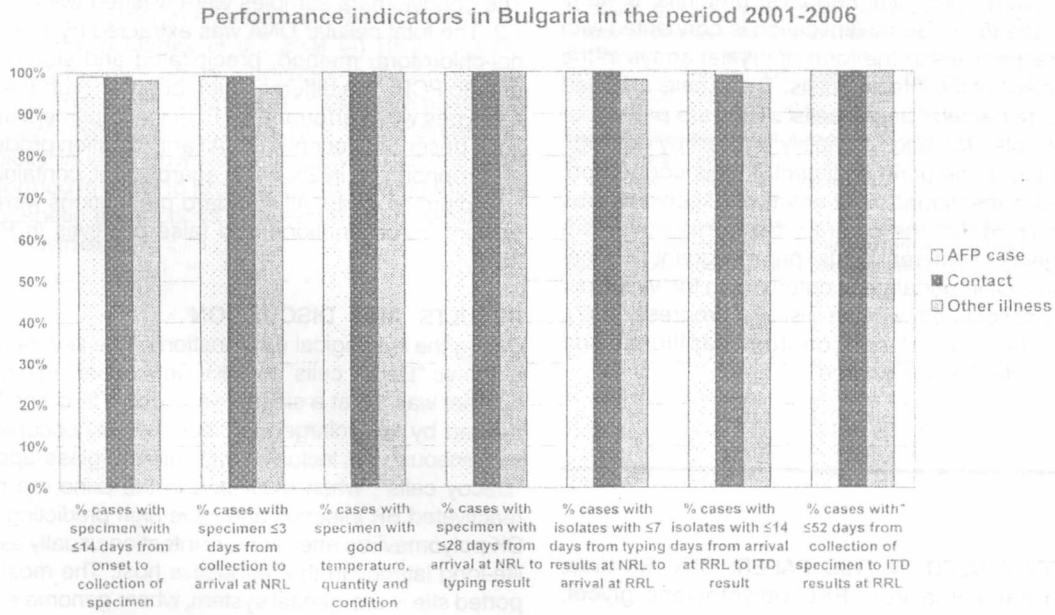
PV – poliovirus
SL – Sabin-like (vaccine) poliovirus

isolated. VDPVs are defined as viruses showing less than 99% VP1 sequence identity to Sabin virus of the same serotype. All our poliovirus isolates were confirmed in WHO Regional Reference Laboratory as Sabin-like ones. The role of various nonpoliomyelitis enteroviruses has been established.

Our results indicate that the AFP virological surveillance is very important for detection of wild poliovirus circulation or the appearance of VDPV.

Today the world is very close to becoming polio-free. Global eradication has continued to be shown as feasible. At this critical final stage of the Global Polio Eradication Initiative the main problems in Bulgaria and in remaining certified polio-free regions in regard to poliomyelitis control are (6):

- Maintain certification-standard surveillance for acute flaccid paralysis
- Ensure highest possible immunity levels against wild poliovirus
- Develop action plans for responding rapidly to importations of wild poliovirus
- Integrate AFP reporting into national surveillance mechanisms to respond to other important diseases.



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ACTIVE BK VIRUS INFECTION IN A PATIENT WITH MULTIPLE-TYPES HPV ASSOCIATED GENITAL LESIONS

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SUMMARY

Human polyoma-(BKV) and papilloma-(HPV) viruses are oncogenic agents, associated with different urological disorders (nephropathies, ureteritis and cystitis) and/or benign or malignant lesions. Here we report on a 45-year-old man, who had suffered from condylomata acuminata and idiopathic uroinfection and present the results on elucidation the etiological agents of the diseases. PCR of the material taken from the lesion, revealed multiple HPV types. Patient's urine was also investigated by cytological staining and pathognomonic "Decoy cells", characteristic for reactivated BK virus infection were found. We discuss eventual pathogenetic relations between both infections.

Key words: BKV, HPV, PCR, Urine cytology

The mixed viral infections are of significant clinical and theoretical importance. After primary contact polyomaviruses establish long-life latency in different organ sites, mainly uro-genital system. Their reactivation is connected in most cases with underlying immune disorder and leads to increased viral replication and cytopahtic pattern of disease. This is associated with various pathologies: nephropathy, interstitial nephritis, urethritis, ureteral stenosis, cystitis (5, 9). BK multiplication is connected with deposing viral particles in the form of crystal arrays in the enlarged nucleus of the infected cells. These cells are shed in urine and are named "Decoy cells". They are pathognomonic for BK infection and are easily defined by cytological examination of the urine sediment. In this connection, they are used in the diagnosis of reactivated polyomavirus BK infection (2, 5). On the contrary papillomaviruses are causative agents of different warts, pre-malignant and malignant lesions. Of importance is determining the viral type, because it is associated with the disease progression (7). Up to now, the data of concomitant papilloma and polyomavirus infections is scarce.

ABBREVIATIONS USED IN THIS PAPER: BKV- BK virus, HPV-human papillomavirus, PEG-polyethylene glycol, PCR-polymerase chain reaction

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MATERIALS AND METHODS

Patient history

A 45 year-old man was initially evaluated for non-specific urethritis at the Clinic of Dermatology and Venereology, Alexandrovska Hospital, Medical University, Sofia. During the examination of the patient verrucous lesions were observed on the glans penis and the perianal area. According to him these lesions appeared approximately two years ago. He had also diabetes mellitus type 2 and elevation of cholesterol and triglycerides.

Urine collection

Second voided urine was collected in plastic containers (Biologix Res. Comp.). The containers were supplied with transportation medium containing absolute alcohol, slowing the bacterial growth (8). The collected urine was immediately sent to the laboratory for cytological analysis.

Condyloma samples

The condylomata tissue was excised from the lesion and preserved in sterile jars with transportation medium (7).

Preparation of glass smears

For the preparation of glass smears was used the semi-automated Bale's method (1). In brief it consisted of the following steps: centrifugation of the urine at 1500 rpm, discarding the supernatant and treating the sediment with 70% ethanol with 2% Polyethylene Glycol (PEG). After this treatment, it was again centrifuged, pipetted and spread on glass slides. They were air dried and fixed in 100% ethanol.

Cytology staining

For the cytological examination routine Szczepanik stain (CYTOCOLOR, Merck KGaA) was applied. It consisted of 10 steps, and was performed according to the manufacturer manual. After finishing the procedure the slides were embedded in Canada balsam and observed by Carl Zeiss light microscope.

HPV detection & typing

The condylomata samples were washed twice in PBS, pH 7.2. The total cellular DNA was extracted by standard phenol-chloroform method, precipitated and stored at -20^0 C prior to PCR. The differentiation between high-risk and low-risk types was performed by PCR using primers and conditions described recently (7). All amplification products were electrophoresed in 2% (w/v) agarose gel, containing 0.5 g/l ethidium bromide. All standard precautions were used to prevent contaminations and false positives in PCR reactions.

RESULTS AND DISCUSSION

During the cytological examination of the sediment pathognomonic "Decoy cells" for BKV were observed (fig. 1). Their number was 5-8 at a single microscopic field and they were defined by the enlarged nucleus, entirely occupied by homogeneous viral inclusion with ground-glass appearance. "Decoy cells", when exfoliated in the urine are marker of reactivated BK infection and have high predicting value (2). BK polyomavirus after primary infection usually establishes life-long latency in the respective host. The most often reported site is uro-genital system, where genome sequences are frequently detected. Under immune impairment or others not-fully elucidated reasons this virus can reactivate and enter into productive infection (5). Diabetes can be a provocative factor for virus reactivation (3). Reactivation is characterized by extensive viral replication and cytopathic destruction of the infected tissue. Thus BK related diseases arise: hemorrhagic and non-hemorrhagic cystitis, interstitial inflammation of the kidney, urethral obstruction and urethritis. In fact, "Decoy cells" shed into urine are from places with active viral multiplication and serve to confirm the diagnosis (2).

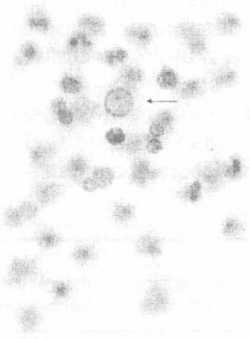

Fig. 1. "Decoy cell" with viral inclusion occupying almost the entire volume of the enlarged nucleus (X100)

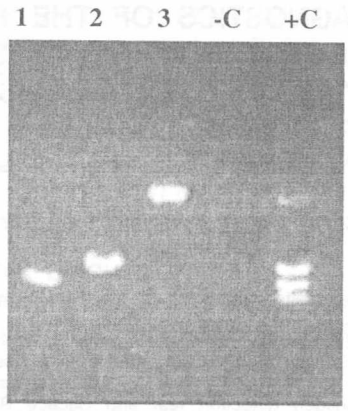

Fig. 2. Gel electrophoresis of the amplified product after HPV PCR. Positive control (+C), HPV-16 (1), HPV-33 (2), HPV-18 (3)

Condylomata accuminata are benign lesions, commonly found in sexually active men and women. Approximately 90% of condyloma acuminata are related to HPV types 6 and 11, which have low oncogenic potential. Rarely, high-risk HPV genotypes can be detected in the condylomatous lesions. We identified multiple HPV infection with both low- and high-risk genotypes (HPV – 16, – 18, and – 33) in the biopsy materials of the patient (fig. 2). Besides, the patient has a history of diabetes mellitus, which has been recognised as an important risk factor for the development of condylomata accuminata and persistence of HPV infection (6). The lesions resolved completely after the treatment with CO_2 laser. No recurrence was detected in the follow up (6 months).

Our results support the assumption that HPV and BKV may complement each other in infecting human uro-genital tissues. According to Martini et al., 2004 (4) BKV, together with HPV, may be involved as a cofactor in the onset/progression of human genital tumors, and raise the possibility that BKV acts synergistically with HPV to enhance their pathogenicity *in vivo*. However, more investigations have to be performed in order to provide better understanding of the pathogenetic interactions between human papilloma- and polyomaviruses.

CONCLUSIONS

With the advances of diagnostic methods, now it is becoming obvious that HPV and BKV can cause mixed infections. The way they interact is not clear, but detection of genome sequences in genital cancers unequivocally involves both of them in the neoplastic transformation of the cell. Nevertheless, more investigations are needed to elucidate their combined role in the different diseases.

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DIAGNOSTICS OF THE FIRST SUSPECT HUMAN CASES OF AVIAN INFLUENZA A/H5N1/ VIRUS IN BULGARIA

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SUMMARY

The first diagnostic investigations have been made, demonstrating avian flu A/H5N1/ virus etiological role. For the period January–March 2006 twenty six suspect patients, having been in close contact with ill or dead birds, and with a subsequent respiratory illness, were tested. Specific A/H5N1/ assays were applied /GeNet Bio rapid antigen detection test and Sacace RT-PCR kit/. Viral isolation was performed using MDCK cell lines and chicken embryos. Avian flu A/H5N1/ virus as a causative agent of respiratory disease was neither isolated, nor detected in any one of the tested patients. Subtype A/H1N1/ human influenza virus, identifiable by HIT and RT-PCR, was isolated in three of the patients tested.

Key words: avian influenza A/H5N1/ virus, diagnostics

The avian influenza (AI), caused by certain highly pathogenic subtypes of influenza A virus in animal populations, and particularly chickens, poses a continual health risk for the human public on a global scale. Direct human infection by avian influenza A (H5N1) virus was first recognized during the 1997 outbreak in Hong Kong. Till April 2006, the cumulative number of the confirmed human cases of avian influenza A/(H5N1) attained 193 cases, of which 109 of lethal outcome. (WHO. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) 11.04.06).

Because of Bulgaria's crossroad location regarding the wild birds' main migratory routes, a real danger of disease transmission exists among the avian population in our country. In the beginning of 2006, A (H5N1) has caused an infection among the poultry in the neighbouring countries / Turkey, Romania, Ukraine/. The virus has been isolated from wild swans in many European countries, Bulgaria including. All these circumstances favor the appearance of the first suspect human cases of avian influenza A(H5N1) in the country. The latter necessitates effective diagnosis not only of human influenza infections, but of avian influenza infections as well. The laboratory identification of influenza virus infections is commonly accomplished by antigen detection, isolation in cell culture, or detection of influenza-specific RNA by (the) highly sensitive and specific reverse transcriptase–polymerase chain reaction /RT-PCR/.(WHO.Recommended laboratory tests to identify avian influenza A virus in specimens from humans. June 2005)

The aim of the survey is the investigation of the first suspect human cases of avian influenza A/H5N1/ virus, using viral isolation and rapid tests of antigen and genome detection.

Table 1.
Suspect A/H5N1/ patients investigated in the laboratory of Influenza for the period January-March 2006

Patient N	sex	age	Town, village/ probable source of contamination
1	f	53	Tutrakan /wild bird
2	f	27	Pleven/ contact with ill bird
3	m	16	Dimitrovgrad /dead bird
4	m	21	Chinese citizen
5	m	39	Varna/dead swan
6	m	45	Shabla/dead swan
7	f	11	Shabla/dead swan
8*#	f	27	Plovdiv/ fast food staff
9#	m	31	Haskovo/dead bird
10	m	16	Shabla/dead swan
11	m	38	Shabla/dead swan
12	f	36	Shabla/dead swan
13	m	48	Turkish citizen
14	f	81	Silistra /dead swan
15	m	58	Silistra /dead swan
16	m	54	Silistra /dead swan
17	f	54	Silistra /dead swan
18	f	38	Shumen/ veterinarian, dead swan
19	m	60	Silistra /dead swan
20	f	22	Silistra /dead swan
21	m	45	Plovdiv/ ill bird
22*	m	49	Vidin / ill bird
23#	m	9	Sliven/dead hen
24	m	36	Dobrich /ill dove
25	m	47	Haskovo/ traveler to China
26	m	8	Gabrovo/ ill bird

• Deceased after severe influenza-like disease
#- Isolated human subtype A (H1N1) viruses A/New Caledonia/ 20/99-like. Identified by HIT

MATERIALS AND METHODS

Clinical specimens: nasal and throat swabs from twenty six patients with influenza like diseases were examined. All the patients were in close contact with ill or dead birds or travellers in countries with registered avain flu cases. Post-mortem lung, tracheal tissue and bronchoalveolar lavage from two patients were also tested. All the samples were collected during the period January –March 2006. Patients' data /sex, age, source of hypothetical contamination/ is shown on Table 1.

Viral isolation was performed by one passage in MDCK and subsequently two passages in chicken embryos. The isolated strains were identified by HIT. The preliminary screening of the samples was made by rapid H5 antigen detection immunosorbent test GeNet Bio.

RT-PCR for detection of viral RNA was performed with the initial samples. RNA was extracted by using monophasic solution of phenol and guanidinium isothiocyanate-Trizol LS (Invitrogen life technologies, USA) an improvement of the single-step RNA isolation method as developed by Chomezynski and Sacchi.

RT-PCR avian Influenza A Virus (H5, H7) Screening and Typing kit (Sacace, Italy) was used for detection of probable A/H5N1/ samples. Conventional RT-PCR for human influenza viruses by using One-Step Ready–to-Go RT-PCR Beads (Amersham Biosciences, U.K) kit for simultaneous cDNA synthesis and PCR reaction was applied. HA and

ABBREVIATIONS USED IN THIS PAPER: NONE

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NA oligonucleotide primers were selected from highly conserved regions of the hemagglutinin and neuraminidase gene segments of the influenza viruses using two specific primer pairs for subtype A (H1N1) and A (H3N2). (Hadzhiolova T., Contemporary methods for influenza virus diagnosis and genetic characterization of influenza viruses, PhD thesis –February 2006, Sofia).
The obtained PCR products (amplicons) were analyzed using 2% agarose gel electrophoresis (100 V for 1 h 30 min.) and their sizes were compared with a DNA molecular weight marker (fX-174 or 100 bp) (Invitrogen life technologies, USA). Positive H5 RNA or standard influenza strains, as well as negative (ddH_2O) controls were included the experiments. The results were read by MiniBis Pro DNR (Bio-imaging Systems, Israel).
In March 2006 we have examined five additional samples received in connection with International Control for human and avian influenza by the Institute for standardization and documentation of medical laboratories (INSTAND). The samples were screened by Directigen Flu A+B (Becton&Dickinson, USA) for detection of viral NP and differentiation of type A or B influenza viruses. Specific H5 antigen detection kit (GeNet Bio, Korea)
and RT-PCR avian Influenza A Virus (H5, H7) Screening and Typing kit (Sacace, Italy) were used for detection of A/H5N1/ samples.

RESULTS AND DISCUSSION

Wnen examining the first suspect A/H5N1/ patients in Bulgaria, the diagnostic scheme stages as developed by the National Influenza Laboratory for human influenza viruses, have been followed. By reason of the disease severity and the necessity of rapid diagnostic response, the scheme was realized as follows:
MDCK cell lines were used for inoculation of the clinical materials immediately following their receipt in the laboratory. Chicken embryos for minimum two consecutive passages have been continuously supplied. Viral isolation has been carried out for 5-7 days.
Instant screening of the initial samples by rapid antigen detection tests and RT-PCR for typing and subtyping of viral RNA.
Despite the usage of two laboratory models, and the performance of three passages, avian influenza viruses were not isolated from any one of the 26 suspect patients' samples. The same negative result for A/H5N1/ was confirmed by the preliminary screening of the initial samples using GeNet Bio rapid antigen detection test, as well as RT-PCR (Sacace, Italy) kit. The sensitivity and specificity of the molecular kit was demonstrated when examining the samples by the International Control, one of the specimens being determined as positive for A/H5/ RNA (Fig. 1).
Influenza strains from three of the patients' samples were isolated (passage in chicken embryos) and identified by

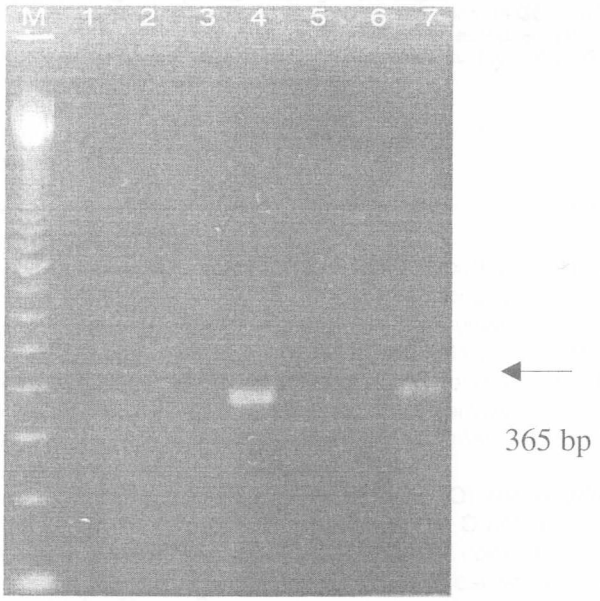

Fig. 1. RT-PCR for detectionof A/H5N1/ avian influenza virus

Legend: Fig. 1

M	DNA100 bp marker
Line 4	(+) sample for avian influenza virus A/H5/
Line 5	(-)control
Line 7	(+)control

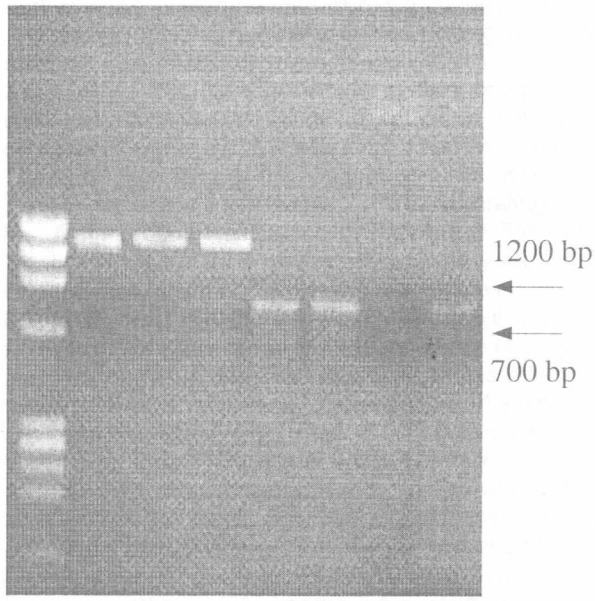

Fig 2. RT-PCR for detectionof A/H1N1/ human influenza virus

Legend : Fig. 2

M	Φx-174 marker
Lines1-3	(+) samples for human influenza virus A/H1/
Line 6	(-)control
Lines 4,5 и 7	(+)samples for human influenza virus A/N1/

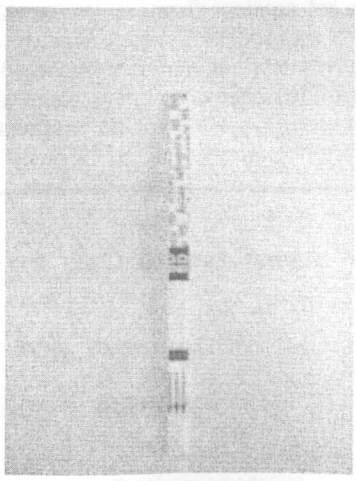

Fig. 3. GeNet Bio rapid A/H5/ test

HIT as human subtype A (H1N1) viruses, A/New Caledonia/20/99-like. RT-PCR using HA and NA specific primer pairs for human influenza viruses also found the same samples RNA positive (Fig. 2).

The results presented here in summarize the preliminary data regarding avian influenza A/H5N1/ virus' diagnostics in humans. We consider RT-PCR (Sacace, Italy) kit adequately effective as regards screening and genotyping of probable A/H5N1/ samples, in spite of the lack of H5 positive clinical sample to the present moment.

The negative result obtained by GeNet Bio (Fig. 3) for the sample received by INSTAND and tested positive by RT-PCR makes us doubt the rapid test sensitivity for avian influenza virus antigen detection in clinical samples.

The investigation of the first suspect A/H5N1/ avian influenza virus patients allowed us to acquire the useful skills when working with highly pathogenic infectious agents, and to gain practical experience when applying the necessary diagnostic methods for viral isolation and influenza H5 antigens and genome detection for extremely short period of time.

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PROFILE OF NEWLY EMERGING MONOPHASIC *SALMONELLA ENTERICA* SUBSP. *ENTERICA* SEROVAR 9,12:L,V:- STRAINS IN BULGARIA CAUSING ENTERIC INFECTIONS

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SUMMARY

Strains of newly emerging in Bulgaria *Salmonella enterica* subsp. *enterica* serovar 9,12:l,v:- causing enteric infections were examined. The biochemical profile of the organisms is consistent with that of *Salmonella enterica* subsp. *enterica* (subspecies I) serovars. All strains showed susceptibility to ampicillin, amoxicillin-clavulanic acid, cephalothin, cefuroxime, cefotaxime, ceftazidime, chloramphenicol,ciprofloxacin, gentamicin, tetracycline, and trimethoprim-sulfamethoxazole. All but one were susceptible to nalidixic acid. On pulsed- field gel electrophoresis (PFGE), the strains showed 15 genomic fragments with one PFGE profile. Causing a cluster of salmonellosis it certainly represents an evolved pathogen of clinical significance. To establish its taxonomy additional genotypic methods are needed.

Key words: Monophasic Salmonella, 9,12;l,v:-, serotyping, phase reversal, PFGE.

INTRODUCTION

Salmonella enterica serovars are important foodborne pathogens of humans causing enteric infections. *Salmonella enterica* subsp. *enterica* (subspecies I) are usually isolated from humans and warm-blooded animals and most of strains isolated in clinical laboratories belong to this subspecies. The most commonly reported serovars causing human infections are *S enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium (1,2).

A serovar is determined by the somatic (O) and flagellar (H) antigens of *Salmonella* organisms. The O antigens determine the grouping, while the H antigens completely define the serovar of a *Salmonella* strain. Most serovars of *Salmonella enterica* subsp. *enterica* (subsp. I) show diphasic variation of flagellar (H) antigens, the alternate expression of which is controlled by a complex switching mechanism (3). Strains expressing both flagellar antigens are called diphasic. However, certain salmonellae express only one type of flagellar antigen, i.e., they are monophasic.

From 24 February 2006 through 17 March 2006 the National Reference Laboratory for Enteric Pathogens, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria received 8 *Salmonella* group D isolates for confirmation. The serotyping results were *Salmonella* 9,12:l,v:-. Knowledge about the occurrence of different serovars in different countries may assist in the recognition and tracing of new emerging pathogens (4).

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We here report the characteristic of these atypical (monophasic) strains that caused enterocolitis in humans.

MATERIALS AND METHODS

Sources of *Salmonella* cultures. *The Salmonella* cultures were obtained for confirmatory testing from two hospitals and three private laboratories in Sofia as isolates from stool specimens. All the isolates were from ill persons – 1 from infant (9 months), 2 from children (5 and 9 years old, respectively), and 3 from adults (20, 23 and 50 years old, respectively). From two of the patients the serovar has been isolated twice – before and after treatment. All these patients suffered diarrhea, abdominal pain, fever and headache. Three of them have been hospitalized. No common food source have been detected and the cases seemed not to be with any epidemiological associations.

Identification of cultures. All serovar 9,12:l,v:- strains were tested biochemically by conventional method. The tests and substrates used (n=15) were as follows: indole, Simmons citrate, hydrogen sulfide, urea agar, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, malonate, beta-galactosidase, glucose, lactose, sucrose, mannitol, maltose and dulcitol.

Serotyping. The *Salmonella* strains were assigned to a serovar 9,12:l,v:- by the slide agglutination method with commercial antisera (S & A Laboratory Reagents, Bangkok, Thailand) according to the Kauffmann-White scheme (5). The strains were shipped to the WHO Collaborating Centre for Antimicrobial Resistance in Foodborne Pathogens, Danish Institute for Food and Veterinary Research, Copenhagen, Denmark where the serovar was confirmed.

Phase reversal typing. The plate method (5,6) was used to express the presence of the second phase H antigen of serovar 9,12:l,v:-. A series of up to 5 repeat tests were performed for each serovar 9,12:l,v:- culture. Weakened or complete inhibition of the H factor (H:l,v) in serotyping were taken to represent successful phase reversal.

Antimicrobial susceptibility testing. Antibiotic resistance tests were performed by agar disk diffusion method (Kirby-Bauer) according to the gidelines recommended by the Clinical and Laboratory Standards Institute (CLSI) (7). The following antimicrobial agents were used: amoxicillin-clavulanic acid, ampicillin, carbenicillin, cephalothin, cefuroxime, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, tetracycline and trimethoprim-sulfamethoxazole (Biomerieux).

PFGE. PFGE was performed using *Xbal* according to CDC PulseNet protocols (8) with a Bio-Rad CHEF DR II apparatus.

RESULTS

Biochemical tests were typical for salmonellae. The strains were indole negative, Simmons citrate and hydrogen sulfide positive, urease negative, lysine- and ornithin decarboxylase positive, phenylalanine deaminase and malonate negative, beta-galactosidase negative, glucose positive, lactose and sucrose negative, mannitol, maltose and dulcitol positive.

None of the strains revealed presence of any second phase H factor.

All strains were susceptible to ampicillin, amoxicillin-clavulanic acid, cephalothin, cefuroxime, cefotaxime, ceftazidime, chloramphenicol,ciprofloxacin, gentamicin, tetracycline, and trimethoprim-sulfamethoxazole. All but one were susceptible to nalidixic acid.

On PFGE the serovar 9,12:l,v:- strains were characterized by 15 genomic fragments. There was not band differences between the strains (Figure 1).

DISCUSSION

There have been a number of reports of monophasic salmonellae in the literature.

Salmonella serovar 4,12:a- was isolated in UK (9,10). Monophasic *Salmonella* serovar 4,[5],12:i- isolates were reported from USA (11,12), Spain (13),Thailand (14). This serovar was implicated as the infectious agent causing food poisoning outbreak in New York (12). *Salmonella* 4,[5],12:i- was among the 5 most common serovars isolated in Thailand from 1993 through 2002 (14).

In Bulgaria 12 monophasic *Salmonella* strains were isolated in 2006: 2 from group C (antigenic formula 6,7:-:5), 1 more from the same group (6,7:c:-), 1 from group B (4,12,:d:-), and 8 strains from group D (9,12:l,v:-).

Salmonella 9,12:l,v:- first isolated in Israel in 1965 has been among the top 10 Salmonella serovars isolated from humans in that country in the following years. It has also been observed frequently in Germany in 1988 and 1989, and infrequently in Switzerland and France (15). This serovar was isolated again in Israel in 2005 from diarrhoeic calves (16). We encounter serovar 9,12:l,v:- in Bulgaria for the first time. Only five salmonellae of serogroup D1 share the l,v antigen: *S.* Mendoza (9,12:l,v:1,2), *S.* Panama (9,12:l,v:1,5), *S.* Kapemba (9,12:l,v:1,7), *S.* Zaiman (9,12:l,v:e,n,x), and *S.* Goettingen (9,12:l,v:e,n,z15). It is interesting that these diphasic serovars are exceedingly rare in our country. *S.* Panama and *S.* Kapemba were isolated in 1956. The next isolation of *S.* Kapemba was in 1998 and it was the last one till now.

Our 9,12:l,v:- isolates have biochemical profile consistent with that of *S. enterica* subsp. I serovars. All the strains are susceptible to antibiotics. On conventional serotyping they were without any second phase H antigen. The phylogenetic origin of serovar 9,12:l,v:- of serogroup D1 that emerged in Israel, Switzerland and Italy has been revealed by molecular typing methods (15). It was shown that the serovar have arisen from an ancestral strain of *Salmonella* of diphasic serovar Goettingen (15). Bulgarian 9,12:l,v:- strains must be compared with serovars Mendoza, Panama, Kapemba, Zaiman and Goettingen both by phenotypic and genotypic methods to establish the serovar under *S. enterica* subsp. I classification.

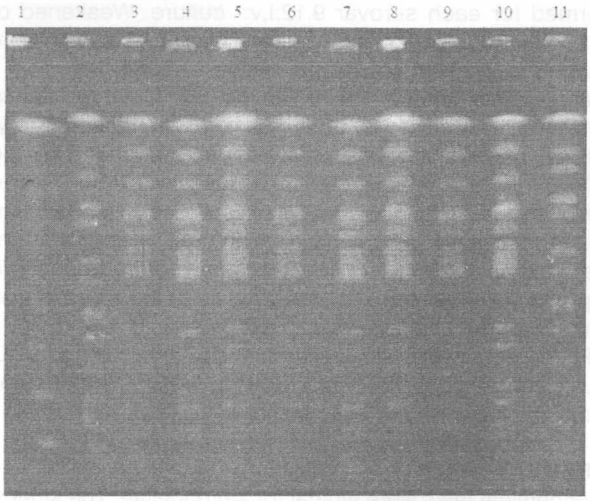

Fig. 1
PFGE profiles of the serovar 9,12:l,v: - strains /lanes 3 to 10/ and serovar Braenderup /lanes 2 and 11/. Marker /lane1/.

On PFGE analysis, the serovar 9,12:l,v:- strains showed homogeneity (Fig. 1) and formed one PFGE profile characterized by 15 bands. These data along with phenotypic data from biochemical investigation and antibiotic susceptibility testing, give us ground to suggest that our 9,12:l,v:- strains have same origin, although patients seemed not to be epidemiologically related and no common food source was detected. We consider this was a cluster of salmonellosis caused by *Salmonella* serovar 9,12:l,v:-.

In conclusion, the first isolation of an atypical (monophasic) *Salmonella* serogroup D1 9,12:l,v:- serovar in Bulgaria is reported. The serovar was characterized biochemically, by antibiotic resistance testing and PFGE profiling. Its potential to cause enterocolitis in humans makes it a pathogen of clinical significance. To establish its taxonomy more genotypic methods are needed.

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CHARACTERIZATION OF SOME VIRULENCE DETERMINANTS OF *AEROMONAS SPP.* ISOLATED FROM MEAT, DRINKING WATER AND PATIENTS IN BULGARIA

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Summary
In the present study 46 *Aeromonas spp.* strains isolated from meat, drinking water and patients in Bulgaria were tested for pathogenicity by studying its hemolysis, enterotoxin production, Congo red absorbtion, neuraminidase activity and cytotoxicity. Hemolysis, tested on sheep erythrocytes, was frequently seen with water isolates and human isolates as with food isolates too. Neuraminidase activity was detected in all *Aeromonas hydrophila* isolates and it was the highest to those *Aeromonas hydrophila* strains isolated from water and meat. Cytotoxicity, evaluated on Vero cells was frequently observed with all meat isolates (100%), with 13 water isolates (65%) and with 10 human isolates (50%). About 60% of the water isolates were positive for enterotoxin activity. These results indicate that the occurrence of *Aeromonas spp.* in the environment represents a potential risk for humans. These results suggest that *Aeromonas* species are potential enteric pathogens in our geographical region.

Key words: Aeromonas spp., water, virulence, pathogenic potential

INTRODUCTION

Aeromonas is an environmental microorganism autochthonous of aquatic environments that can be sporadically transmitted to humans (Austin and Adams, 1996; Borrell et al., 1998).

In humans, *Aeromonas spp.* are associated with life threatening diseases (Janda and Abbott, 1998). Moreover, the *Aeromonas* species is one of the important agents associated with diarrhea (Bundell, 1983). *Aeromonas spp.* have been identified as one of the important food-borne pathogens (Handfield et al., 1996). *Aeromonas spp.* are able to survive and multiply at low temperatures in a variety of food products stored between -2 and 10°C such as beef, roast beef, and pork (Rossi Junior et al., 1996), and can produce virulence factors even at these low temperatures (Majeed et al., 1989). A number of putative virulence factors (aerolysin/hemolysin, proteases, DNases) that may play an important role in the development of disease in human have been described in several species of the genus (Howard et al. 1996; Pemberton et al. 1997; Soler et al. 2002; Castro-Escarpulli et al. 2003). In spite of a significant number of virulence determinants produced by *Aeromonas spp.* their association with gastroenteritis is not clearly linked (Sechi et al. 2002).

46 *Aeromonas spp* strains were isolated from drinking water, food and patients with diarrhea (14 children, mostly infants and 6 adults) from May 2004 to the end of June 2005. The strains were identified as *Aeromonas hydrophila* (n=26), *A. caviae* (n=16) and *Aeromonas sobria* (n = 4). The objectives of this study was to determine the spreading of virulence determinants among *Aeromonas spp.* isolated from meat, drinking water and patients in Bulgaria, and to study some virulence factors of the isolates from different origin.

MATERIAL AND METHODS

Bacterial strains

Forty six motile *Aeromonas spp.* were isolated over a period of a year from meat (6 strains), drinking water (20 strains) and patients with diarrhea (20 strains). Identification of the genus *Aeromonas* was done using standard tests including motility, Gram staining (-), cytochrome oxidase (+), D-glucose fermentation (37°C/24 h) (+), catalase (+) and sensitivity to O/129 (-) (Sigma St. Louis, MO, USA) after 24 h at 30°C. Voges-Proskauer test was performed to identify *A. hydrophila* and *A. caviae*. When the results of VP test were negative, these strains were identified as *A. caviae*.

Phenotypic identification

Before each test, all the cultures were grown on tryptose soya agar (TSA) (Oxoid) at 37°C for 24h. Growth 6,5% sodium chloride was used to discriminate *Aeromonas spp.* from *Vibrio fluvialis*. The species were identified by 15 conventional specific assays chosen from those described by Popoff (1984), such as esculin hydrolysis, D-arabinose utilization, gas production from glucose, indole, acetyl methyl carbinol production, H_2S formation from cysteine and the capacity to grow under oxidative and fermentative conditions. The strains were re-identified by Vitek AutoMicrobic System (Vitek ASM, bioMerieux Vitek, France) at the Department of Microbiology - National Center of Infectious and Parasitic Diseases - Sofia. Distribution of species was the following: twenty six *A. hydrophila* isolates (ten of them were isolated from patients); sixteen *A. caviae* isolates (ten of them were isolated from children with a diarrhea) and four *A. sobria* isolates.

Phenotypic determination of virulence factors

Five colonies of those grown on TSA were suspended in 2 ml phosphate buffer saline, pH 7,2. The density of each suspension was adjusted to 0,5 of the McFarland standard ($1,5x10^8$ cells ml^{-1}). We used 100 µl of this suspension for the phenotypic determination of virulence factors. All strains were tested in duplicate.

Preparation of bacterial culture supernatants

Aeromonas were cultured in 20 ml of trypticase soy broth (Difco Laboratories, Detroit, MI, USA) by shaking (100 rpm) (New Brunswick Scientific Co.) for 24 h at 37°C. Culture supernatants were collected by centrifugation (10000Ч*g* for 30 min at 4°C) and filtered through a 0.22-mm membrane filter (Millipore Corp., Bedford, MA, USA). The filtrates were stored at -70°C until assayed.

Enterotoxin production

The suckling mouse test modified by Burke et al. (1981) has been used as a model for the detection of *Aeromonas* enterotoxins. All 46 *Aeromonas* isolates recovered from clinical and environmental sources were cultured in TSB-YE: Tryptone Soya Broth (Oxoid) supplemented with 0,6%(w/v) of Yeast Extract (Oxoid), as recommended by Burke et al.(1984). Culture supernatants, containing a drop of 2% Evans Blue solution were inoculated intragastrically to three suckled Balb/C mice (2-5 days old). After inocula-

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tion the mice were maintained at 25°C. After 2 hours for the detection of the enterotoxin action, groups of mice were killed. Intestines were removed from the remaining bodies and both intestinal weights (IW) and remaining body weights (BW) were measured for each mice group and the ratios (IW/BW) calculated. We considered as enterotoxigenic strains the ones that originated ratious =/ > 0,08 (Burke et al., 1987). Supernatantes without bacteria, acting as negative controls, originated always ratios under 0,06. Culture supernatantes from strains considered as toxigenic were heated at 56°C for 10 min in water bath and tested in mice to verify the effect of temperature in the enterotoxic activity. All *Aeromonas* toxigenic supernatants lost their activities after heating at 56°C for 10 min.

Cytotoxicity to Vero cells

The cytotoxicity assay was performed as described by Konowalchuk et al. (1977). Vero (African green monkey kidney) cells were cultivated in tissue culture flasks with Eagle's modified essential medium (MEM; Gibco, Grand Island, NY), supplemented with 10% fetal calf serum, 0.75 mM L-glutamine, 40 mg/ml gentamicin and 1 mg/ml amphotericin B. Confluent monolayers were removed with trypsin EDTA, resuspended to approximately 4 x 10^5 cells/ml in MEM and 0.1-ml samples were pipetted into each well of a 96-well microtiter plate. After incubation at 37°C in 5% CO_2 for 72 h, the medium was replaced with 180 µl MEM and 20 ml of the bacterial filtrate was added to each well. Cytotoxic activity on Vero cells was observed by exposing a monolayer of the cell to serial dilutions of the filtrates. After incubations at 37°C for 24 h in 5% CO_2, destruction of at least 50% of the Vero cells was recorded as a positive result. As negative controls, some wells received only MEM, TSB or *E. coli* K12/711 filtrates and *E. coli* H30 filtrates were used as positive control. Each sample was tested in triplicate. Vero monolayer morphology was observed under the inverted microscope and checked for cytotoxic effect for five days.

Congo red dye uptake

The ability to take up Congo red dye was determined on agar plates supplemented with 50 мg/ml of Congo red dye. Five microliters of each suspension was streaked onto the plates and incubated at 37°C for 24 h. Orange colonies were considered as positive. Different intensities in the dye uptake were expressed as + and ++ (Paniagua et al., 1990).

Hemolytic activity

Hemolytic activity was determined as a clear zone of β-haemolysis around the colonies on blood agar plates containing 5% (v/v) of sheep blood after a 24 h incubation at 37°C (Gerhardt et al., 1981).

Neuraminidase activity

Neuraminidase activity was established quantitatively according to Aminoff (1961). One unit of neuraminidase activity is defined as the amount that releases 1 мg of N-acetylneuraminic acid for 1 min under standard condition using glucomacropeptide as a substrate (Abrashev et al., 1979).

Table 1. Phenotypic characteristics of *Aeromonas* isolates.

Characteristic	*A. hydrophila* n= 26	*A. sobrian* =4	*A.caviaen* =16
Motility	+	+	+
H_2S from cysteine	+	+	+
Oxidase production	+	+	+
Indole production	+	+	+
Glucose fermentation	+	+	+
Salicin fermentation	+	-	+
Acid from D-mannitole	+	+	+
Arabinose utilisation	+	-	+
Acetoin production	+	+	-
Esculin hydrolysis	+	-	+
Lysine decarboxylase	+	+	-
Ornithine decarboxylase	-	-	-
Congo red absorbtion	+	+	+

+ , positive for 70% of isolates; -, negative, i.e. positive<30% of isolates
All *Aeromonas caviae* isolates were lysine-decarboxilase negative.

RESULTS

Biochemical characteristics

Some of the phenotypic characteristics of the investigated *Aeromonas* isolates are presented on Table 1. It was found that all investigated strains are ornithine decarboxilase negative. Acetoine production was not found among *Aeromonas caviae* strains. Esculin hydrolysis was usually found associated with *A. hydrophila* and *A. caviae.* Production of lysine decarboxylase was associated with *A. hydrophila* and *A. sobria.*

Congo red absorbtion

91% of the strains were positive for uptake of Congo red, although with a different intensity, as 45% of them produced orange colonies and 46% reddish orange colonies.

Extracellular hemolytic activity

The quantification of the extracellular hemolytic activity (Table2) indicated that out of 28 *Aeromonas hydrophila* isolates, 24 strains (85,7%) were β-hemolytic of which 10 strains (41,6%) had been isolated from diarrheal cases. All *Aeromonas sobria* strains were β-hemolytic too. All *Aeromonas caviae* isolates were nonhemolytic on 5% sheep blood agar.

Enterotoxin production

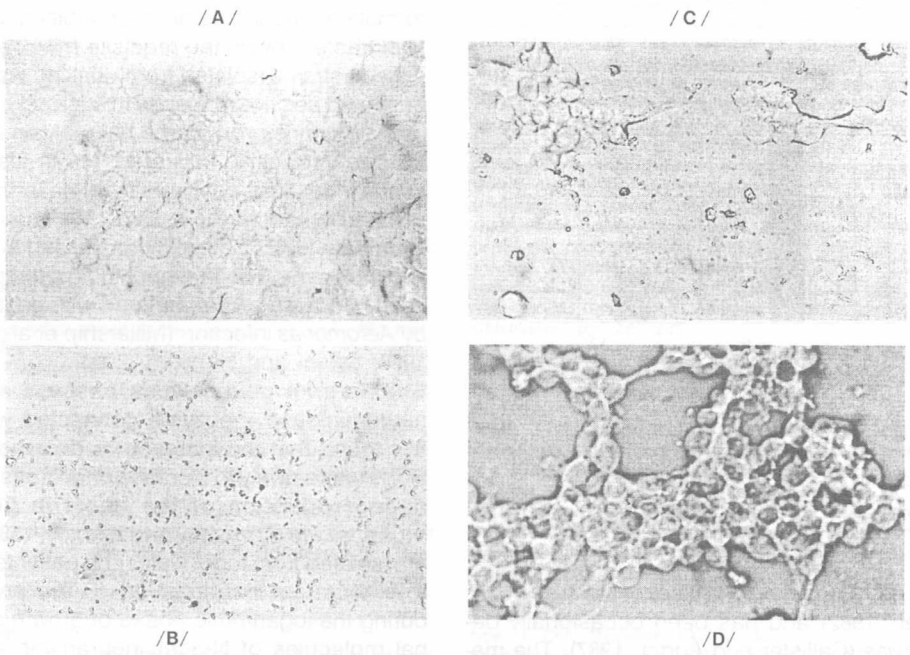
With the objective of verifying the enterotoxigenic level of *Aeromonas spp.* strains, isolated from meat, drinking water and patients with diarrhea 46 strains were tested (28 from *A. hydrophila*, 14 from *A. caviae* and 4 from *A. sobria*), using suckling-mouse test (Table 2). Producers of enterotoxins were found in three (50%) strains of *A. hydrophila,* originated from meat knife, hands, before the person begin working, and from ready to consume deboned meat, in nine (75%) strains of *A. hydrophila* isolated from drinking water, in ten (100%) strains *A. hydrophila* originated from patients, in three (75%) strains *A. sobria*, isolated from drinking water, and in two (20%) strains of *A. caviae*, isolated from patients. 57,69% of environmental strains were enterotoxigenic compared to 60% of clinical samples enterotoxic to suckling mouse model.

Table 2. Percentage hemolytic, cytotoxin-positive and enterotoxin-positive *Aeromonas spp.* isolates from different origin.

Organism	Source	Number ofIsolatestested	Hemolysis[a]	Number of cytotoxin-positive isolates[b]	Number of enterotoxin-positive isolates
A.hydrophila	meat	6	β-hemolysis5 (83,3%)	6 (100%)	3(50%)
	water	12	β-hemolysis9(75%)	8 (66,6%)	9 (75%)
	patients	10	β-hemolysis10 (100%)	10 (100%)	10 (100%)
A. caviae	water	4	Nonhemolytic	0 (0%)	0 (0 %)
	patients	10	Nonhemolytic	0 (0%)	2 (20%)
A.sobria	water	4	β-hemolysis4 (100%)	4 (100%)	3 (75%)

[a] Hemolytic activity to 5% sheep blood agar
[b] Cytotoxic to Vero monkey kidney cells

Figure 1. Cytotoxic effect on Vero cells 24 h after exposure to *Aeromonas hydrophila* broth filtrates. /A/Toxine-negative *A.hydrophila* isolate(no change from control cells); /B/ cytotoxin-positive *A. hydrophila* isolate (cell death manifested by pyknotic nuclei, loss of adherence, cell shrinkage); /C/ *A. hydrophila* isolate producing rounding effect and loss of adherence; /D/ cytotoxin - positive *A. hydrophila* isolate



Cytotoxicity for Vero cells

Cytotoxicity, evaluated on Vero cells was observed with all meat isolates 6 (100%), with 12 water isolates (60%) and with 10 human isolates (50%) (Table 2). Cytotoxin activity and visual Vero cells damages after incubation at 37°C for 24h in 5% CO_2 are presented at micrograms on Figure 1. Cytotoxin titres and percentage of isolates cytotoxic to Vero cells are presented on Figure 2. Cytotoxicity was demonstrated by none of *A. caviae* isolates.

Neuraminidase activity

All strains were analyzed for neuraminidase production and no enzymatic activity was detected in 13 of them (Table 3). All investigated *A. hydrophila* strains, 2 *A. sobria* strains and 5 *A. caviae* strains demonstrated neuraminidase activity at different range. These strains was found to secrete neuraminidase into the culture medium which contained $1,3 \times 10^5$ to $1,3 \times 10^6$ colony forming units per milliliter after 24 hours of cultivation. The strains isolated from water and meat demonstrated higher neuraminidase secretion compared with that of the strains isolated from patients.

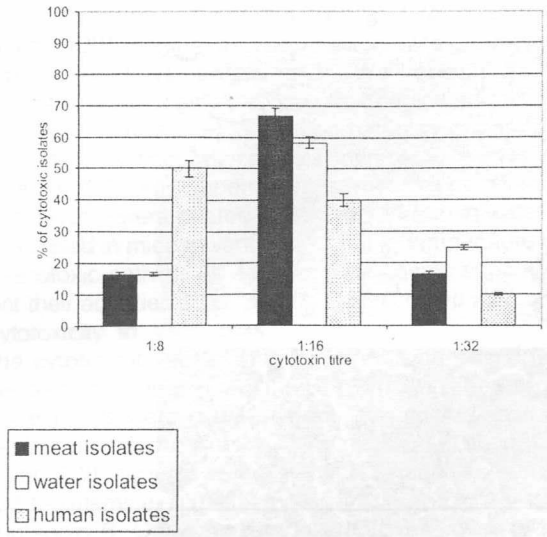
DISCUSSION

Forty six isolates of *Aeromonas spp.* were isolated from drinking water, food and patients in Bulgaria. The aeromonads isolated in the present study mainly belonged to three species – *A. hydrophila*, *A. sobria* and *A. caviae*, which are often reported to be associated with human disease.

Biochemical identification by routine methods indicated that all aeromonads produce acid from mannitol and are ornithine negative. Among the various phenotypic traits examined, esculin hydrolysis was usually found associated with *A. hydrophila* and *A. caviae* and production of lysine decarboxylase with *A.hydrophila* and *A.sobria*. Possesion of three or more characteristics such as ability to produce acetyl methyl carbinol, lysine decarboxylase, high hemolysin titre, inability to ferment arabinose are reported to be associated with enterotoxin production (Kirov et al., 1986). Reindetification of the strains by Vitek AutoMicrobic System demonstrated that the most prevalent species isolated from drinking water were *A. hydrophila* but we found several *A. caviae* and *A. sobria* strains too. The study revealed that *Aeromonas spp* occur in the potable and domestic drinking water supplies and even in the chlorinated water supplies in Bulgaria. Only *A. hydrophila* strains were isolated from meat samples. The environmental isolates were found to be positive for virulence factors. Several studies have demonstrated that many mesophilic aeromonads isolated from drinking water and food can exhibit toxigenic factors. Millership et al. (1986) found that cytotoxicity was demonstrated by 28% of *Aeromonas* isolates (mainly A. *hydrophila*) from chlorinated and unchlorinated drinking water but by none of the strains of *A. caviae* (which represented 50% of the isolates). Our results supported that finding. Notermans et al. (1986) found that all of 26 drinking water isolates of *A. hydrophila* and 9 of 22 isolates of *A. sobria* exhibited hemolytic enterotoxin and cytotoxicity to Vero cells. According to the same authors none of 14 isolates of *A. caviae* was cytotoxic and hemolytic. We have found that many of the strains isolated from drinking water and food show properties identical to those of clinical isolates. For example, 75% of all water samples and 50% of meat samples were enterotoxigenic. 100% of *A. hydrophila* strains isolated from patients were enterotoxigenic, compared to 75% of *A. hydrophila* strains isolated from drinking water. According to Burke et al.(1984) many of the strains *Aeromonas hydrophila* found in water have properties identical with those of the clinical isolates. Suckling mouse activity was described in fish isolates (Boulanger et al., 1977) and in a variety of human and environmental isolates (Janossy and Tarjan 1980; Johnson and Lior, 1981). Finding of potentially pathogenic organisms in drinking water and food indicate that *Aeromonas spp.* may be hazardous to public health. Isolation of aeromonads from the water samples of the city water supply possibly indicates inadequacy of chlorination of the water supplies or resistance of aeromonads to chlorination (Alavandi and Ananthan, 2003).

Species of *Aeromonas* are capable of expressing number of extracellular toxins and enzymes (Gosling, 1996; Howard et al., 1996). Species of *Aeromonas* also produce a range of cell-surface and secreted proteases which probably enhance virulence (Glosing, 1996). To date, cytotoxin production among aeromonads has been described mainly

Figure 2. Percentage and cytotoxin titre of *Aeromonas spp.* isolates



for *A. sobria* and *A. hydrophila* (Barer et al., 1986; Champsaur et al., 1982) and has been occasionally described for *A. caviae* (Callister and Agger, 1987). The majority of *A. hydrophila* and *A. sobria* isolates in our study produced both cytotoxic, enterotoxic and hemolytic factors, and poses neuraminidase activity. Cytotoxicity, evaluated on Vero cells was observed with all meat isolates 6 (100%), with 13 water isolates (65%) and with 10 human isolates (50%). Aslani and Hamzeh (2004) reported that 67,9% of diarrheal strains showed vero cytotoxic activity that had a significant association with diarrhea. Vero cells are reported to be the most sensitive cell line for detection of *Aeromonas* cytotoxin (Barer et al., 1986). Handfield et al. (1996) found that cytotoxicity, evaluated on seven cell lines, was frequently observed with food isolates (92%) and with water isolates (73%). Cytotoxin production correlated also with a positive lysine decarboxilase phenotype or a positive Voges-Proskauer phenotype (Cumberbatch, 1979).

The production of hemolytic toxins has been regarded as strong evidence of pathogenic potential in aeromonads. However, nonhemolytic aeromonads have been implicated as human pathogens (Namdari and Bottone, 1990).

We found that 69,2 % of the environmental samples (*A. hydrophila* and *A. sobria*) were hemolytic to sheep erythrocytes. This result confirm data reported by other authors who observed that hemolytic *Aeromonas spp.* are isolated in non-polluted environments (Gibbotti et al., 2000). None of *A. caviae* isolates demonstrated hemolytic activity to sheep erythrocytes. According to some investigators sheep erythrocytes are less sensitive than erythrocytes from other mammals (Albert et al., 2000; Schubert., 2000). However, there is information about β-hemolytic *A.caviae* isolates recovered from water (Alavandi and Ananthan, 2003). Ten of our *A. caviae* isolates were obtained from children, mainly infants with diarrheal ilnesses. According to Namadri and Bottone (1990) *A. caviae* as well as *A. hydrophila* and *A. sobria* is a bona fide enteric pathogen especially among formula-fed neonates or other subjects whose gastrointestinal tracts provide the requisite milieu for survival.

Fifteen strains isolated from drinking water and food demonstrated positive response in suckling mouse model. Most of that strains produced в-haemolysin too and were cytotoxic to Vero cells. Fujii et al. (1998) also reported that *A. sobria* induced a positive response in the suckling mouse test and in haemolytic assays. We found that all clinical *A. hydrophila* isolates were cytotoxic and also virulent in suckling mouse model. In other studies, the production of cytotoxin has been also correlated with gastroenteritis caused by *Aeromonas* infection (Millership et al., 1986, Wong et al., 1996, Aslani and Hamzeh, 2004).

Strains from genus *Vibrio* possess ability to produce neuraminidase – enzyme connected with pathogenicity. Itŷs found that the increase or decrease of the virulence leads respectively to increase or decrease of the neuraminidase production of the bacteria like *Erysipelothrix rhusiopathiae* (Abrashev and Nikolov, 1984). *Aeromonas* that are isolated from food, water and patients have been shown to elaborate a neuraminidase. The enzyme is secreted during the logarithmic phase of growth and cleaves terminal molecules of N-acetylneuraminic acid (NANA) from glycoproteins and gangliosides (Rafelson, 1963). According to our experiments the strains isolated from water and meat demonstrated higher neuraminidase secretion compared with that of the strains isolated from patients. According to some investigators (Lichtensteiger and Vimr, 1997) neuraminidase takes part in bacterial survival in the environment as an important tool for nutrition (Corfield, 1992). The role of the sialic acid rich mud in the survival of *Aeromonas* in the natural aquatic systems has been observed (Abrashev and Orozova, 2004).

The virulence of several enteropathogenic bacteria such as *Yersinia enterocolitica* (Farmer et al., 1992) and *Vibrio cholerae* (Nataro and Kaper, 1998) has been correlated with the ability of the isolates to take up Congo red dye. 91,3% of the strains studied showed the ability for binding Congo red in agreement with date reported by other authors in *Aeromonas* (Statner and George, 1987; Palumbo et al., 1989; Paniagua et al., 1990; Castro-Escarpulli et al., 2003). According to Ishiguro et al.(1985) Congo red uptake test may be a good virulence marker of *A. salmonicida*. But according to Statner and George (1987) Congo red uptake may not be a useful marker for virulence of motile *Aeromonas* species. Our data confirmed that standpoint.

In conclusion, the incidence of *Aeromonas spp.* in chlorinated drinking water and food should be surveyed, in relation to *Aeromonas*-associated enterocolitis. In Bulgaria, there have been no studies on *Aeromonas* and its putative virulence factors. The present work therefore highlights an important incidence of *Aeromonas spp.* with virulence potential and antimicrobial resistance, isolated from water, meat and patients in Bulgaria. Many of the environmental

Table 3. Absolute values and percentage of neuraminidase positive *Aeromonas* species isolated from water, meat and patients in Bulgaria.

Strain	Origin	Samples	Positive samples (%)	Number of strains with neuraminidase activity 1,5-10U/ml	Number of strains with neuraminidase activity10-20U/ml	Number of strains with neuraminidase activity20-70 U/ml
A. hydrophila	water	12	12 (100%)	1	7	4
A. caviae	water	4	3 (75%)	2	1	-
A. sobria	water	4	2 (50%)	2	-	-
A. hydrophila	meat	6	6 (100%)	1	4	1
A. hydrophila	patients	10	10 (100%)	1	8	1
A. caviae	patients	10	2 (20%)	2	-	

strains show properties identical with those of the clinical isolates. We suggest that such strains in water and food may be potential enteric pathogens.

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ANTIMICROBIAL RESISTANCE OF *P. AERUGINOSA* ISOLATES FROM DIFFERENT WARDS IN VARNA UNIVERSITY HOSPITAL OVER A 3-YEAR PERIOD

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SUMMARY

During the period 2002- 2004, 272 nonduplicate *P.aeruginosa* strains, isolated from different clinical specimens, were studied. The identification was done by conventional methods, semiautomated and automated tems. The strains were tested to Piperacillin (PIP), Piperacillin/ tazobactam (TZP), Ceftazidime (CAZ), Cefepime (FEP), Amikacin (AM), Ciprofloxacin (CIP), Imipenem (IMP) and Meropenem (MEM). The most active antimicrobials for the studied period were: TZP>IMP>PIP>FEP>AM>CIP>CAZ. During 2004, the resistance of *P.aeruginosa* showed an important increase against CAZ, but significant decreases in resistance were noted for AM and CIP during the same period. IMP resistance has stayed in constant levels. Among 247 isolates, MEM and IMP showed similar activity. For the period 2002-2004, multi-resistant (resistance to at least 4 of the tested drugs) were 11.4 % of the strains. The agent with the lowest level of resistance among multi-drug resistant isolates was IMP (34.5%).

Key words: P.aeruginosa, *antimicrobial resistance, multi-drug and cross resistance*

INTRODUCTION

P.aeruginosa is one of the most important nosocomial pathogens, which is a leading cause of mortality, particularly among patients with immunosuppression, malignancy, cystic fibrosis, burn or traumatic wounds (6,7). The spectrum of human infections caused by *P.aeruginosa*, ranges from skin infections to fulminant sepsis. Nosocomially acquired *P.aeruginosa* isolates tend to be more resistant to antimicrobials than do community-acquired strains, frequently displaying resistance to multiple classes of antimicrobials. Development of resistance may occur during antimicrobial therapy and is particularly well documented during monotherapy. The present study investigated the antimicrobial susceptibility and recent trends of antimicrobial resistance of isolates *P.aeruginosa* from non-ICUs and ICUs patients in Varna University Hospital from 2002 to 2004.

MATERIALS AND METHODS

Hospital "St. Marina" is a University Hospital in Varna, Bulgaria with 962 beds. During the research period in the clinical microbiology laboratory, a total of 272 nonduplicate strains *P.aeruginosa*, isolated from different clinical specimens, were studied. *P.aeruginosa* isolates were identified using standard microbiological procedures, automated (Bactec 9120, Sceptor/BD), and semi-automated (Crystal/ BD, Lachema/Czech Republic) systems. Resistance to the following antimicrobial agents: Piperacillin (PIP), Piperacillin/tazobactam (TZP), Ceftazidime (CAZ), Cefepime (FEP), Gentamicin (G), Amikacin (AM), Ciprofloxacin (CIP), Imipenem (IMP) and Meropenem (MEM), was assessed using Disc Diffusion method, strictly following NCCLS recommendations. *P.aeruginosa* isolates were considered to be multidrug resistant (MDR) if the isolate was resistant to at least four of the tested antipseudomonal drugs.

RESULTS AND DISCUSSION

Resistance rates of *P.aeruginisa* isolates for the studied period are shown on Table 1.

Although increase in the resistance rates (TZP, CAZ, FEP) for this three year period was detected, it is statistically not significant ($P>0.01$). But resistance rate of *P.aeruginosa* isolates declined significantly for Ciprofloxacin and Amikacin ($P<0.01$). Resistance to Imipenem was stable for these 3 years and was under 10%. Among 247 simultaneously tested isolates, Meropenem and Imipenem showed similar susceptibility rates, Imipenem being slightly more active – 85.4%, against 82.59%.

Decreasing susceptibility was observed with greatest declines for Ceftazidime. This increasing resistance may be correlated to higher usage of the drug in the hospital. Similar rate of resistance to Ceftazidime, was detected in a global study, conducted in USA (1999-2002), but they report much higher rates of resistance to Ciprofloxacin and Imipenem (3). Piperacillin/tazobactam represents the beta-lactam with the lowest rate of resistance, with 93% of all isolates remaining susceptible in 2004. The data showed that the resistance rates to Piperacilli/tazobactam, Imipenem, Cefepime and Ciprofloxacin were lower, compared to results from similar study, performed in other University Hospital in Bulgaria (1). Our combined 2002-2004 results demonstrated relatively high rates of susceptibility to Piperacillin/tazobactam, Imipenem, Piperacillin and Cefepime (table 1).

Infections due to MDR *P.aeruginosa* require aggressive treatment, usually involving at least two antimicrobial agents with synergistic activity. Even more, some authors write that such infections might be treated with a synergistic combination of antimicrobials to each of which strains are resistant (8). Multi-drug resistance is a well-known phenomenon and its mechanisms have been studied extensively in the last years (2,4,5). Table 2 summarizes the contribution of Piperacillin, Piperacillin/ tazobactam, Ceftazidime, Cefepime, Amikacin, Ciprofloxacin and Imipenem resistance to MDR phenotypes for clinical isolates of *P.aeruginosa* from 2002 to 2004, tested with all these 7 agents.

Among all *P.aeruginosa* isolates, 59.5% showed no resistance to any of the seven antibiotics analyzed. Resistant to all seven antibiotics were 1.5% of the isolates, which probably is a result of combined mechanisms of resistance. Resitance to 5 antibiotics was found in 3.3%, while Fluit et al reports 6.8% (4). In spite of this that the combination treatment is generally recommended for suspected MDR *Pseudomonas aeruginosa* infections, there is a risk that this approach may encourage resistance to multiple agents (5).

ABBREVIATIONS USED IN THIS PAPER:
PIP-Piperacillin, **TZP**-Piperacillin/tazobactam, **CAZ**-Ceftazidime, **FEP**-Cefepime, **AM**-Amikacin, **CIP**-Ciprofloxacin, **IMP**-Imipenem

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Table 1
Annual and 2002-2004 combined resistance (%) of 272 isolates of P.aeruginosa collected from ICU_s and non-ICU_s patients to 7 antimicrobial agents

ANTIBIOTIC	PIP	TZP	CAZ	FEP	AM	CIP	IMP
2002 - 2003	14,76	4,54	18,17	12,49	28,4	31,81	9,08
2004	10,32	7,05	26,08	16,29	15,76	18,47	9,77
2002 - 2004	11,76	6,24	23,52	15,07	19,84	22,78	9,55

PIP-Piperacillin, **TZP**-Piperacillin/tazobactam, **CAZ**-Ceftazidime, **FEP**-Cefepime, **AM**-Amikacin, **CIP**-Ciprofloxacin, **IMP**-Imipenem

resistance, 91,4% from the MDR isolates were resistant to Amikacin. The possible resistance mechanisms to aminoglycosides are enzymatic inactivation by aminoglycoside-modifying enzymes, efflux pumps, loss of cell permeability or combined mechanisms. The agent that showed the lowest level of resistance among MDR isolates was Imipenem (34.5%). Table 4 presents the cross-resistance to 7 selected antibiotics in the 272 *P.aeruginosa* solates. Combined 2002-2004 results demonstrated that Amikacin resistant *P.aeruginosa* isolates also showed high

Table 2
Contribution of resistance to individual antimicrobials to MDR phenotypes among clinical isolates of P.aeruginosa in 2002 – 2004

Number of againts to which izolate are resistant	N-%		N-% resistant													
			RIP		TZP		CAZ		FEP		AM		CIP		IMP	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
0	162	59,5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	42	15,4	1	2,4	0	0	21	50	3	7,1	1	2,38	7	16,6	9	21,4
2	22	8	3	14	1	4,54	4	18	1	4,5	14	63,6	15	68,2	0	0
3	15	5,51	5	33	1	6,6	4	27	6	40	13	86,6	12	80	4	26,6
4	13	4,77	7	54	5	38,4	11	85	13	100	8	61,5	8	61,5	1	7,6
5	9	3,3	5	56	2	22,2	8	89	8	89	9	100	8	88,8	4	44,4
6	5	1,8	5	100	5	100	4	80	5	100	4	80	4	80	2	40
7	4	1,47	4	100	4	100	4	100	4	100	4	100	4	100	4	100

MDR were 11.4% of all isolates (mostly originated from ICUs).The most frequent MDR phenotype was resistance to Ceftazidime, Cefepime, Amikacin and Ciprofloxacin, while Flamm et al reports that the most frequent MDR phenotype detected was resistance to Gentamicin, Ciprofloxacin and Tobramycin (3). Cefepime and Amikacin resistance were the two most common drug resistance phenotypes found in MDR isolates (table 3).
Resistance to beta-lactams (incl. extended-spectrum cephalosporins) is associated with overexpression of the chromosomal AmpC beta-lactamases, extended-spectrum betalactamases (ESBLs), impermeability of the outer membrane and increased efflux. As concerns aminoglycosides levels of cross-resistance to Ciprofloxacin, but also Piperacillin, Piperacillin/tazobactam and Cefepime resistant isolates often showed high levels of cross-resistance. The clinical interpretation of this resistance must be taken into account when a combination of antipseudomonal drugs is used for treatment of severe *P.aeruginosa* infections.
In summary, for the studied three year period there was a loss of activity in some antibiotic groups (Piperacillin/tazobactam, Ceftazidime, Cefepime). Ciprofloxacin and Amikacin showed significant trend of increasing their activity. In the beta-lactam group Piperacillin/tazobactam was most active, followed by Imipenem. The increasing resistance to beta-lactams during the years needs attention.

Table 3
Contribution of resistance to individual antimicrobial agents in MDR clinical isolates of P.aeruginosa, collected from 2002 to 2004

YEARS	MDR isolates		PIP	TZP	CAZ	FEP	AM	CIP	IMP
	N	%	%	%	%	%	%	%	%
2002-2004	31	11,4	72.4	48,3	86,2	96	91,4	97,6	34,5

MDR - Total number and percentage of isolates associated with MDR (resistance to >= 4 of the 7 antimicrobial agents).

Table 4
Resistance against pairs of antibiotics in P.aeruginosa isolates (n = 272)

First antibiotic	Perfentage (N) resistant to each antibiotic separately		% of Resistant strains testefirst antibiotic, resistant to a second antibiotic						
	N	%	TZP	CAZ	FEP	AM	CIP	IMP	PIP
RIP	32	11,76	50	65,6	65,6	68,7	59,3	15,6	100
TZP	17	6,2	100	82,3	76,4	52,9	47	35,2	100
IMP	25	9,5	20	36	36	60	48	100	24
FEP	39	15	33	61,5	100	69,2	64,1	20,5	53,8
CAZ	64	23,5	21,8	100	42,1	37,5	42,1	10,9	31,2
AM	54	19,85	16,6	46,2	46,2	100	90,7	25,9	40,7
CIP	62	22,78	12,9	40,3	40,3	79	100	19.3	30,6

PIP-Piperacillin, **TZP**-Piperacillin/tazobactam, **CAZ**-Ceftazidime, **FEP**-Cefepime, **AM**-Amikacin, **CIP**-Ciprofloxacin, **IMP**-Imipenem

Our data suggests that currently the choice of a carbapenem, Cefepime, or Piperacillin/tazobactam in combination with Amikacin would give the widest potential empirical activity against *P.aeruginosa*. Relatively low is the percent of MDR isolates for the period 2002-2004. Multi-resistance and cross-resistance are a major problem in *P.aeruginosa*. Regular monitoring of antimicrobial resistance trends for the most important hospital pathogens, one of which is *P.aeruginosa*, is of great importance for an adequate hospital antibiotic policy.

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A CASE OF LYME CARDITIS WITH ATRIOVENTRICULAR BLOCK AND REVIEW OF THE LITERATURE

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SUMMARY

A case of a 39-year old patient with Lyme carditis manifested as atrioventricular block grade III was presented. Carditis occurred in the absence of erythema migrans and the patient did not recall a tick bite, only a skin inflammation about a month ago and arthralgia now. He was hospitalized because of dizziness, presyncope and bradycardia. ELISA showed significant levels of IgM antibodies to *B. burgdorferi*. After treatment with Famotidine, Methylprednisolon and Rocephin, the AV block evolved through grade II to grade I. Serology was more positive after the second 15 day-course with Rocephin. At that time the patient felt only significant weakness but had no cardic abnormalities. Specific antibodies decreased 2 months later and after a 25-day course with doxycycline p.o. The patient was clinically healthy and had no subjective complaints. Lyme carditis cases are usually underestimated not only in our country. The principal manifestations of Lyme carditis are conduction abnormalities, pericarditis, myocarditis, tachyarrythmias, and congestive heart failure. They should be considered as possible Lyme borreliosis even in patients without anamnesis of tick bite and erytema migrans rash and Lyme serology should be ordered. Reliable diagnosis and adequate early treatment are guarantee for avoiding complications and progression of the disease.

Key words: Lyme borreliosis, Lyme carditis, Borrelia burgdorferi, AV block

INTRODUCTION

Lyme borreliosis is a systemic disease caused by the spirochete, *Borrelia burgdorferi*, and transmitted by *Ixodes ricinus* tick. The disease occurs in three stages. Stage 1, or the early-localized stage, develops within 30 days after the initial tick bite and is notable with erythema migrans rash and/or a flulike illness. Stage 2, or the early-disseminated stage, occurs weeks to months after the first stage symptoms and is characterized by neurological, rheumatologic, skin or ocular complications and musculoskeletal pain. Cardiac abnormalities usually become manifest at this time. Stage 3, the late Lyme borreliosis, occurs several months to years after the first stage and is characterized by oligoarthritis affecting the large joints or neurological symptoms (10).

CASE REPORT

We describe a 39-year old patient with Lyme carditis manifested as atrioventricular block grade III. Carditis occurred in the absence of erythema migrans and the patient did not recall a tick bite, only a skin inflammation about a month ago and arthralgia now. He was hospitalized (July 2006) in Gabrovo Hospital because of dizziness, presyncope and bradycardia. Electrocardiography showed AV block grade III and ventricle frequency of 25/min. Echocardiography showed slightly dilated cardiac chamber size, slightly decreased chamber function and mitral valve insufficiency grade I. A serum sample for *B. burgdorferi* serology was drawn. ELISA showed significant levels of IgM antibodies to *B. burgdorferi* (value of 30,29 U/ml – cut off 24 U/ml) and no IgG antibodies. Treatment with Famotidine, Methylprednisolon and Rocephin was started before appearance of *B. burgdorferi* serological results. The AV block evolved through grade II to grade I and ventricle frequency trough 50/min to 55/min (fig.1). A new 15 day-course with Rocephin was prescribed (August 2006). Fifteen days after the end of the therapy with Rocephin, a second serum sample was tested (September 2006). Both classes of anti-*B. burgdorferi* antibodies – IgM and IgG increased their levels (IgM – 57,33 U/ml; IgG – 102,10 U/ml). At that time the patient felt only significant weakness but had no cardiac abnormalities. A course with doxycycline p.o. for 25 days was prescribed (October 2006). Third serology was drawn 15 days after the end of this therapy (November 2006). It showed slight decreasing of the specific antibodies (IgM 35,29 U/ml; IgG – 83,60 U/ml). The patient was clinically healthy and had no subjective complaints.

As a discussion, we may say that Lyme carditis cases are usually underestimated not only in our country. That is why, reports on Lyme caridits cases are relatively rare in the world literature. Rhythm and conductive disturbances of cardiac function are very typical for Lyme carditis and should be considered especially when the patients have a history of tick bite or erythema migrans lesion. Patients often can not describe properly the skin lesion as was the case with our patient but still such skin inflammation in combination with cardiac abnormalities about a month after was considered and Lyme serology has been ordered. One interesting point in the serological results is continual increasing of the specific antibodies despite adequate treatment and clinical improvement. This is a typical finding in the course of treated Lyme disease. It could be explained by specific features of antibody production in Lyme borreliosis - delate appearance and continuous persistence.

Lyme borreliosis serology usually positivates late in the course of the disease. That is why, negative serologic findings do not rule out the diagnosis. Serological tests can be negative in the first 6 to 8 weeks of disease. On the other hand, positive serologic findings are usually long-lasting. Specific IgM antibodies are normally found up to 1 year after the recovery and IgG are present much longer. Serologic testing cannot be evidence for antibiotic treatment independently. Positive serology solely, in the absence of clinical symptoms, is not a criterion for therapy.

REVIEW OF THE LITERATURE

Steere at al (9) first described the cardiovascular manifestations of Lyme disease in 1980 with a report of 20 North American cases. European cases have been reported since 1984 when Houwerzyl et al (3) and Cornuau et al (1) described the first cases. Now, it is estimated that 4% to 10% of patients in the United States with untreated Lyme disease develop carditis. The incidence is less in Europe – between 0,3% and 4% (6). Some authors suggest that the North American incidence is overestimated because one series of 187 patients with stage 2 disease, characterized

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by neurological manifestations, had no clinical evidence of cardiac involvement (2). Another prospective study of 61 patients with early-localized Lyme disease found only one patient that developed atrioventricular block (7).

Lyme carditis usually occurs shortly after tick bite or erythema migrans with a range of 4 days to 7 months (5). The cardiac manifestations most commonly occur with other features of Lyme borreliosis (arthritis, erythema migrans or neurological disease) but also may occur independently. The patient may not recall the rash or the tick bite.

Common complaints are light headache, syncope, dyspnea, and palpitations. In a review of CDC of 84 patients with Lyme carditis, 69% reported palpitations, 19% had conduction abnormalities, 10% had myocarditis, and 5% had left ventricular systolic dysfunction (6).

tients with idiopathic **dilated cardiomyopathy** and 12.7% of patients with coronary artery disease had positive ELISA values (8).

Endomyocardial biopsy specimens from two other European patients with clinical **congestive heart failure** gave also positive results (4). Both had complete resolution of myocardial dysfunction after treatment with penicillin.

Culture of *B. burgdorferi* and clinical improvement of heart failure after antibiotic treatment in seropositive patients have not been reported from the US.

The different outcomes of these studies may be due to differences in patient populations or in the serologic assays used. Nevertheless it seems prudent to consider *B. burgdorferi* infection in the differential diagnosis and cause of dilated cardiomyopathy.

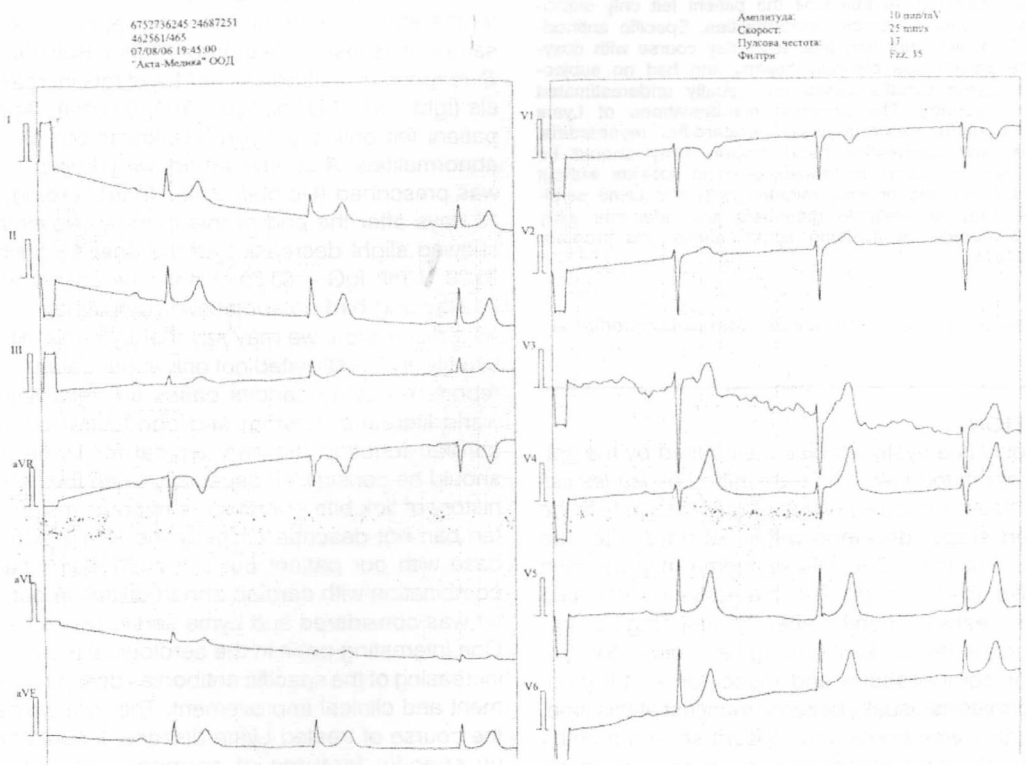

Fig.1. Electrocardiography of the patient with Lyme carditis.

The most typical manifestation of Lyme carditis is **atrioventricular block**. This conduction disturbance is fluctuating, and patients may vary from having a prolonged P-R interval to complete heart block within minutes. The most common clue to cardiac involvement is alternating tachycardias and bradycardia. Lyme borreliosis can affect the conduction system diffusely, but the atrioventricular node seems to be the most vulnerable. Complete heart block resolves within 1 week, and lesser conduction disturbances resolves within 6 weeks.

Myocardial involvement by Lyme carditis is the presence of diffuse ST and T wave changes, seen in up to 65% of patients. Myocardial involvement leading to cardiomegaly, left ventricular dysfunction, or clinical congestive heart failure is thought to occur in 10% to 15% of patients with Lyme carditis. In most cases, myocardial dysfunction is mild and self limited.

According to one study of European group, 26.4% of pa-

As a conclusion, the principal manifestations of Lyme carditis are conduction abnormalities, pericarditis, myocarditis, tachyarrythmias, and congestive heart failure. They should be considered as possible Lyme borreliosis even in patients without anamnesis of tick bite and erytema migrans rash and Lyme serology should be ordered. Reliable diagnosis and adequate early treatment are guarantee for avoiding complications and progression of the disease.

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DETECTION OF PARODONTOPATHOGENS IN A DENTAL PLAQUE USING FISH METHOD

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SUMMARY
The aim of the author was to study the FISH method in order to apply this modern method in Bulgaria. FISH method was used for a detection of parodontopathogens in the dental plaque for first time in our country. The samples from 8 patients with periodontal disease were tested with double stranded DNA probes for some of the most frequent parodontopathogens: Actinobacillus actinomycetemcomitans, Prevotella spp, Porphyromonas gingivalis, Fusobacterium nucleatum, Tannerella forsythia, Veillonella spp., Selenomonas spp., Campylobacter spp.

Key words: Parodontopathogens, FISH Method

It is now well established that caries and periodontal diseases are infectious diseases associated with resident microorganisms of the dental plaque (1). Periodontitis involves the destruction of the connective tissue attachment and the adjacent alveolar bone (2). The induction and progression of periodontal tissue destruction is a complex process involving plaque accumulation, release of bacterial substances, and host inflammatory response (1,3,4). Although bacteria rarely invade tissues, they may release substances that penetrate the gingivae and cause tissue destruction directly, by the action of enzymes and endotoxins, or indirectly, by induction of inflammation(5). In deciding upon therapy it is important to isolate the microorganisms in the dental plaque

STUDY POPULATION

Eight patients, aged 30-40 years, with periodontal disease, periodontal pockets of>4mm and radiographic evidence of localized bone loss. They had not received any periodontal treatment or antimicrobial medication during the previous 6 months.

ABBREVIATIONS USED IN THIS PAPER: Fluorescent in sith Hybridization-FISH

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Clinical Examination and Collection of Plaque Samples

Two weeks before sampling, probing depth is recorded and documented at six points per tooth. Subgingival plaque samples are obtained from two selected deep pockets by inserting one paper point for 10 sec in each pocket, carefully avoiding any contact of the paper point with supragingival plaque or oral fluid (6). The tips of the paper points are transferred to 1 ml of reduced transported fluid and 20% glycerol in Eppendorf vials and stored at –45°C, then transported deep-frozen to the host laboratory three months after sampling.

FISH Technique

Fluorescent in situ hybridization (FISH) is employed to detect directly in plaque samples the presence of the bacteria using 16S rRNA probes (7). The probes are short oligonucleotides (double stranded DNA), conjugated with fluorochromes Cy3 or FAM, recognizing species- or genus-specific regions of the target - 16S rRNA.

100ml from the sample is centrifuged and resuspended in an equal volume of 0,9% NaCl to eliminate the glycerol, used for cryopreservation. Then the required aliquot is diluted with CB (0,9%NaCl/ 0.02%NaN_3/ 0,00025% CTAB) and strongly vortexed. 24-welled microscope slides are coated with 10ml in each well from the diluted with CB samples, prepared for the IF-assay. Drying at a room temperature and fixation in paraformaldehyd at 4°C for 20min follows. To avoid the wall fluorescense the slides are coated

Table 1

Sample N	tooth	PD mm	FISH CFU/ml
1.1	16	5	$3,3.10^5$
1.2	46	5	$3,3.10^5$
2.1	31	5	$2,4.10^5$
2.2	25	6	$6,3.10^5$
3.1	41	5	$2,6.10^5$
3.2	15	5	$4,7.10^5$
3.3	31	5	$2,3.10^5$
4.1	41	7	$1,8.10^5$
4.2	21	4	$2,5.10^4$
5.1	32	5	$1,2.10^5$
5.2	16	5	5.10^4
5.3	27	5	$4,7.10^4$
6.1	47	7	$1,9.10^5$
6.2	16	4	2.10^4
6.3	41	4	$1,2.10^4$
7.1	11	4	$1,1.10^5$
7.2	12	4	4.10^5
7.3	27	4,5	2.10^6
8.1	26	4	$3,4.10^5$
8.2	*31*	*4*	*1.10^4*

Table 2

Probe	Microorganism
Act639-Cy3	A.actinomycetemcomitans
Pdent654-FAM	P.denticola
Pnig655-FAM	P. nigrescens
Pint652-Cy3	P.intermedia
Ptan630-Cy3	P.tannerae
Fnuc233c-Cy3, FUS664-FAM	F.nucleatum
VEI217-FAM, SPO623-Cy3	Veillonella spp.
Pend740-Cy3	P.endodontalis
Pgin1006-Cy3	P.gingivalis
Tfor127-Cy3, Tfor582-Cy3, Tan1260a-Cy3, Tan1260b-FAM	T.forsithia
SPO623-Cy3	Selenomonas spp
Camp655-Cy3	*Campylobacter spp*

Table 3

N'	1		2		3	4	5	
probe	Act639-Cy3	Pdent654-FAM	Fnuc233c-Cy3	VEI217-FAM	Pgin1006-Cy3	Tfor127-Cy3	Tan1260a-Cy3	Tan1260b-FAM
11								
12			6,6.10^3			4,7.10^3	1,2.10^3	5,4.10^3
21			4,8.10^4			1,1.10^4		
22			3,3.10^4					
31			2,0.10^4			2,6.10^3		
32								
33			4,0.10^4					
41		10^4	2,1.10^5					
42								
51			3,3.10^5					
52			1,6.10^3					
53								
61			3,3.10^4				1,8.10^3	
62								
63								
71			3,6.10^3					
72			3.10^3					2.10^3
73			2.10^4			9,4.10^3		
81								
82								

N'	6	7	7.1 Selen.	7,2 Veilonella	8	9	10	11
probe	Pint652-Cy3	Pnig655-FAM	SPO623-Cy3	Camp655-Cy3	FUS664-FAM	Tfor582-Cy3	Pend740-Cy3	Ptan630-Cy3
11			1,1.10^5				3.10^4	2,4.10^3
12								
21								
22			1,3.10^4					
31			5,6.10^4					
32			3,3.10^5					
33			2,9.10^4					
41			4,4.10^3	5.10^3				
42			3,7.10^4					
51			2.10^3					
52			1,4.10^3					
53								
61			9,6.10^3	10^4				
62								
63								
71				8.10^3				
72			8,6.10^3	5.10^3	8.10^3	3.10^3		
73			2.10^4		2,2.10^4			
81								
82								

with 6ml per well Denhardt's solution, diluted 1:50 in 0,9% NaCl, and incubated at 37°C for 1h. After the incubation the drops are aspirated, the slides are rinsed with nanopure water and air-dried. Next each well is covered with 6ml probe solution, diluted with buffer (25% formamide) 1:10. The slides are incubated at 46°C for 2h and immediately dipped after that into washing buffer at 48°C for 20 min. Again the slides are rinsed with nanopure water and air-dried; finally mounted with mounting fluid.

RESULTS

The results are described in the tables.

From each patient are taken 2 or 3 samples. The teeth-pockets, where samples are taken from, and the probing depth (PD) are represented on table 1. It is also shown the total microbial count determined using EUB probe.

Table 2 represents the probes and the corresponding bacteria. For the detection of Veillonella spp., F.nucleatum and T.forsithia were used different nucleotide sequences. The probe SPO623-Cy3 was used for the detection of both species Veillonella and Selenomonas, because of their different shape.

Table 3 shows the number of the sample, the number of the well on the slide (N'), the probes in each well for the different microorganisms and their microbial count CFU/ml. If conjugated with different fluorochromes two probes can be added in one well. With Cy3 the cells shine in orange and with FAM in green. F.nucleatum was found in seven patients with the probe Fnuc233c-Cy3, but in one using the probe FUS664-FAM. Selenomonas spp also was found in seven patients. Tree patients were positive for Veillonella spp. with the probe SPO623-Cy3. T.forsithia was found in four patients with the probe Tfor127-Cy3, in two when the probes Tfor582-Cy3 or Tan1260a-FAM were used and in one when Tfor582-Cy3 was used. Campylobacter, P.endodontalis and P.denticola were found only in one patient each.

DISCUSSIONS

FISH as method is fast specific, cheap and culture independent. With this method single cell can be identified. The disadvantages are on one hand that the reading of the slides is tiring and intensive and on the other to some degree subjective. It has a lower detection limit (ca. 10^4) slightly higher than best selective culture or PCR procedures. From the results is obvious that the probes have to be improved and the background fluorescense should be eliminate in order to eliminate the subjective factor.

The data should prove valuable for the treatment of aggressive periodontitis in Bulgaria and the comparison between the conventional and modern methods of analysis will be useful to estimate which is appropriate for application in the local laboratories (8,9).

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SPECIFYING CLINICAL FORMS OF TOXOCAROSIS AFTER TESTING SEROLOGICALLY SUSPECTED PATIENTS

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SUMMARY

Toxocarosis is helminthic disease with various clinical pictures. Few years ago the main clinical forms were visceral and ocular. Recently some authors separated neurological, skin form and toxocarosis of mussels and disseminated in immunocomprommised patients. The aim of the study was to determine clinical forms of toxocarosis in Bulgaria after testing sera samples from suspected patients in ELISA and Western blot. The results showed that in suspected patients from our country is possible to obtain all described clinical forms of toxocarosis. Patients with visceral form were the largest number while tested patients from groups with other clinical features were not sufficient for radical conclusions. When the results were confirmed by Western blot the number of positive patients from all tested groups was significant lower than tested in ELISA. These differences could be explained with cross reactions due to other helminthic diseases.

***Key words**: toxocarosis, larva migrans, clinical forms, ELISA, Western blot.*

Toxocarosis is parasitic disease with prolonged course and polymorphic clinical signs due to the migration in the human body of larvae of intestinal roundworms of dogs *(Toxocara canis)* and cats *(Toxocara cati)*.

Two major clinical forms of toxocarosis were described 5-6 years ago–visceral (visceral larva migrans) and ocular (ocular larva migrans) (4, 13). Recently despite these two forms were also classified neurological form and some more rare forms as a toxocarosis of mussels, skin and disseminated toxocarosis in immunocompromised patients (1).

The classic visceral toxocarosis, includes one or more of the following clinical signs: hepatosplenomegaly, fever, pulmonary involvement, eosinophilia (over 20%), leucocytosis and hypergammaglobulinaemia. At present different allergic manifestations like urticaria, angioedema, oedema Quincke are often connected with the classic visceral form (5). Ocular form is well known by literature data since it has been described (13).

The separation of the new proposed neurological form is based on the fact that a specific organ - the brain is the final site of the Toxocara larval migration. CSN involvement may exhibit of different clinical signs - meningitis, meningoencephalitis. The relationship between epilepsy and Toxocara infection is still discussed (3, 11).

Toxocarosis of mussels was described lately in tropical areas while the skin form was connected clinically by authors with symptoms like pruritus, alopecia, eczema (1, 8). The aim of this study is to determine clinical forms of this parasitosis in Bulgaria among suspected for toxocarosis patients with positive results by serological testing in ELISA and confirmed by Western blot.

MATERIALS AND METHODS

I. Materials:

1. Sera samples: In our study 1184 sera samples from patients suspected of toxocarosis were tested. Specimens were collected from all over the country for 8 years (1997-2005). 240 from tested sera were from children till 18 years of age and 944 from adults. The main clinical features of these two groups of patients are presented on table 1.

II. Methods:

1. Enzyme linked immunosorbent assay (ELISA): At the NCIPD diagnostic ELISA kit for obtaining IgG toxocara antibodies, based on excretory-secretory (E/S) antigen from second stage larvae of Toxocara canis was elaborated. Polystyrene plates were coated with E/S antigen and incubated 18 hours at 2-8°C. Then plates were washed three times with PBS - Tween 20. Tested sera were diluted 1:200 with PBS - 0.5% BSA - Tween 20. After incubation at 37°C for 30 minutes followed by three fold washing, plates were incubated for the same period with goat human-anti IgG conjugated with peroxidase. The reaction was visualized by substrate orthophenilendiamin (OPD) in the presence of 0.01% hydrogen peroxide. The optical density was read at 492 nm by Reader Uniscan (Labsystems). For ELISA results interpretation a detection limit of 0.4 extinction value was used to discriminate between positive and negative results.

2. Western blot: Toxocara E/S antigen has been resolved by SDS-electophoresis into polypeptide bands and transferred by electro blotting onto nitrocellulose membrane (0.45 μm) (Western blot procedure). The membrane was washed with buffer, containing Tris-NaCl, and cut into 0.2 mm ready to use strips. For the immunoenzyme reaction strips were incubated 18 hours at 4°C with tested sera samples from patients diluted 1:400, washed again and incubated with the conjugate (human anti-IgG conjugated with peroxidase for 3 hours at room temperature. After third step washing the complex antigen-antibody was detected by addition of substrate (diaminobensidine - DAB + 0.05% hydrogen peroxide) and the results (presence of specific anti -Toxocara IgG) were read by the obtained coloured fractions. According to Magnaval et al. (1991) specific for Toxocara are antigenic fractions with low molecular weight corresponding to 24-35 kDa.

RESULTS AND DISCUSSION:

The results after ELISA implementation showed that 129 from all 1184 sera samples were serologically positive for toxocarosis. When these positive sera samples were tested by W.B. in 83 cases the result was confirmed. Nevertheless the largest number positive specimens were from patients with symptoms for visceral form of toxocarosis. All tested sera samples from patients suspected for visceral toxocarosis were 1085. Positive result in ELISA we obtained in 116 (10.4%) of them whereas W.B. confirmed this result in 77 specimen. According to the clinical, laboratory data and positive serological result distribution of patients is presented on fig.1. The most frequent symptoms among positive for visceral toxocarosis were clinically manifested allergy, eosinophilia as a leading clinical sign, gastrointestinal and respiratory symptoms.

The highest seropositivity has been established among

ABBREVIATIONS USED IN THIS PAPER:

ELISA - Enzyme linked immunosorbent assay
W.B. -Western blot

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patients with eosinophilia (15.2% in ELISA, 10.7% in W.B.), in both children and adult groups. These facts confirm the statements of other authors that eosinophilia is characteristic for toxocarosis and when there is a lack of other clinical symptoms toxocarosis should be considered (11).

In our study a relation to T.canis was proved in patients with clinically manifested allergy with unclear etiology of illness in 7.8% in ELISA and 5.0% in W.B. which was described from other authors (5).

The number of tested patients with clinical data for classical signs of toxocara infection like pulmonary symptoms (pneumonia) (43) and hepatomegaly (89) was comparatively little than patients with allergy and eosinophilia. After

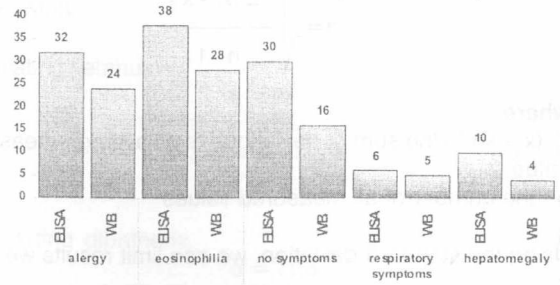

Figure 1.
Distribution of positive patients with visceral toxocarosis in ELISA and Western blot according to clinical diagnosis

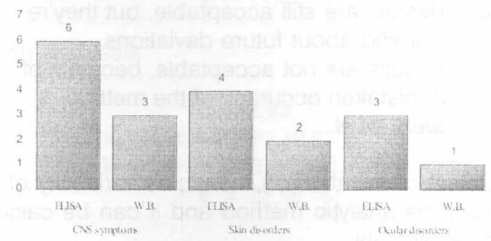

Figure 2.
Distribution of patients positive for neurological, ocular and skin toxocarosis

of the positive in the group with data for CNS involvement - 31.6% in ELISA, 15.7 by W.B Neurological toxocarosis was not well studying there were published 12 cases of CNS involvement between 982 and 2002 (12). A case of toxocaral meningitis in Bulgaria was reported in 1998 also (7). According to our results toxocarosis maybe considered among large part of patients with neurological symptoms especially in the differential diagnosis of eosinophilic meningitis and epilepsy (fig.2).

Patients with symptoms for ocular form of toxocarosis were a total of 21, as the positive among them were 14.2% in ELISA and 4.8% by W.B. There was a described case of ocular toxocarosis in Bulgaria (1998) (2). That is why when horioretinitis, uveitis etc. occur, toxocarosis maybe considered.

The clinical symptoms for patients with skin form of the disease were mainly alopecia and pruritus. Although the tested number of sera samples were not too much, the comparatively high relative part of the positive among them (17.4% in ELISA, 8.7% by W.B.) shows that toxocarosis should be considered in the differential diagnostic plan and in such contingents.

Our research did not present statistically significant difference in toxocarosis seropositivity between children and adult as well between men and women ($p > 0.05$).

The most commonly used method for immunodiagnosis of toxocarosis nowadays is ELISA for detection of anti Toxocara antibodies. W.B. was elaborated in 1991, but despite of assignment of specific polypeptide fraction with low molecular weight (24-35 kDa) for diagnostic purposes this method is used in a few laboratories (9). The comparative analysis of results after testing patients with toxocarosis with ELISA and W.B. showed that W.B. is more specific. That is seen from results obtained in our study where the relative part of positive patients tested for confirmation with W.B. was remarkably low. Particularly it was obvious among

The purpose of the present study was to specify clinical

Tab.1 Clinical features of suspected patients tested for toxocarosis

Clinical features	Patients under 18		Patients over 18		Total
	Boys	Girls	Men	Women	
Allergy - urticaria, oedema, asthma	28	57	134	256	475
Respiratory symptoms - cough, wheeze, pneumonia	10	2	17	14	43
Hepatomegaly, splenomegaly, renal disorders	6	2	39	42	89
Eosinophilia (till 65%)	29	32	80	122	263
No symptoms	27	20	95	109	251
CHS involvement - epilepsy, meningitis	2	7	5	5	19
Skin disorders - dermatitis, pruritus, eczema	4	3	8	8	23
Ocular disorders - chorioretinitis, iridocyclitis, uveitis	5	6	4	6	21
Total	111	129	382	562	1184

testing in ELISA the results showed high relative part of seropositive among these groups - 14% from patients with respiratory disorders and 11.2% from these with gastrointestinal symptoms. Western blot confirmed 12% and 5% from obtained positive specimen in these two groups. We could not complete categorically conclusions about the frequency of toxocarosis among people with these symptoms because number of tested patients was not significantly high.

The group of patients without clinical signs numbered 251 and positive result in ELISA we obtained in 12% of them as in W.B. the relative part was 6.3% (fig.1). Asymptomatic toxocarosis was described often by other authors according to whom 3-12% from positive for these parasitosis patients showed no clinical features (6, 10).

In patients with clinical data for other forms of toxocarosis the number of tested sera samples was comparatively low (a total of 99 sera samples). The highest is the relative part

forms of toxocarosis in Bulgaria after testing suspected patients. Results from this first survey give general information for frequency among patients with different clinical forms of toxocarosis and could be useful base for medical specialists for purposeful search of this parasitosis.

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INVESTIGATION ON THE POTENCY TEST OF BACTERIAL COMBINED VACCINES PRODUCED IN BB-NCIPD LTD

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Bul-Bio NCIPD

SUMMARY

Whole animal assays for potency of bacterial combined vaccines provides very important information for their activity. Nevertheless their expense, the large number of animals used, the time spend and their variability, the whole animal assays for potency and process of establishing of accuracy, precision, specificity, linearity and reliability are performed for the final product release and stability testing. The information is of a great importance, because of the ability to estimate even the procedure of potency testing is correct and the results are between the limits of precision performance. It is possible the data are acceptable, but at the level that gives a warning for eventual future problems or the precision of the test is not satisfactory. On the basis of revue of the potency test results for a long period the levels of acceptance of the results for each component of the combined vaccines, produced in Bul Bio - NCIPD Ltd., are established. In the study all produced lots are included – Tetatox since 1996, Tetadif since 1998, Diftet since 1997 and Diftetkok since 1997. This information gives permission for making a decision about improving the process.

Key words: bacterial vaccines, tetatox, tetadif, diftet, diftetkok

INTRODUCTION

Bioassays are very variable in outcome and also often use animals in the assay which in themselves are variable, and can have broad acceptance limits. Whole animal assays are more difficult and involve the care, maintenance and handling of animals. They are time consuming and highly variable. The biological response of an appropriate species to an active drug is compared to the response to a reference product as a measure of activity. The validation of the potency test of bacterial vaccines is the process of establishing of accuracy, precision, specificity, linearity and reliability. Information permits making a decision about better performing of the process.

MATERIALS AND METHODS

Vaccines for prophylactic purposes, produced in Bul Bio – NCIPD Ltd.

Potency test

Calculating the average value of all measurements, which gives the "basic tendency" for the results:

$$\bar{\bar{x}} = \frac{\Sigma x_i}{n}$$

where:

x_i - every measured value

n - number of tests

Calculating the standard deviation (y) shows the variations around the average value. This is used as limiting value, when getting the results by any analytic method.

Standard deviation can be calculated using the formula:

$$\sigma = \sqrt{\frac{\Sigma (x_i - \bar{\bar{x}})^2}{n-1}}$$

where:

$\Sigma (x_i - \bar{\bar{x}})^2$ – the sum of deviations of all average measured value

n - the number of all measured values

Using the standard deviation, we can limit results we get:

$\bar{\bar{x}} \pm 1\sigma$ Results of measurement are acceptable and the method is under control

$\bar{\bar{x}} \pm 2\sigma$ Results are still acceptable, but they're warning about future deviations

$\bar{\bar{x}} \pm 3\sigma$ Results are not acceptable, because of a mistaken accuracy of the method ***alert level***

Coefficient of variation (v) measures variability of results, got from the analytic method and it can be calculate by using the formula:

$$v\,(\%) = \frac{\sigma}{\bar{\bar{x}}} . 100$$

where:

σ – standard deviation

$\bar{\bar{x}}$ – average value of results

RESULTS

TETATOX

Calculation and statistic analysis:

σ =11,59

$x \pm 1\sigma$ = 79,81-102,99

$x \pm 2\sigma$ = 68,22-116,58

V = 12,7 %

DIFTET

Calculating tetanus

σ =17,7

$x \pm 1\sigma$ = 89,9 – 125,3

$x \pm 2\sigma$ = 72,2 - 143

V = 16,45 %

Calculating diphtheria

σ = 17,3

$x \pm 1\sigma$ = 58,10 – 92,70

$x \pm 2\sigma$ = 40,8 - 110

V = 22,90%

ABBREVIATIONS USED IN THIS PAPER: NONE

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TETADIF

Calculating tetanus

σ =15,6

$x \pm 1\ \sigma$ = 72,4 – 103,6

$x \pm 2\ \sigma$ = 56,8 – 119,2

Calculating diphtheria

σ = 2,72

$x \pm 1\ \sigma$ = 10,09 – 15,53

$x \pm 2\ \sigma$ = 7,37 – 18,25

V = 21,23 %

DIFTETKOK

Calculating tetanus

σ =26,1

$\bar{x} \pm 1\ \sigma$ = 83,9 – 136,1

$\bar{x} \pm 2\ \sigma$ = 57,8 – 162,2

V = 23,72 %

Calculating diphtheria

σ = 11,8

$\bar{x} \pm 1\ \sigma$ = 56,4 - 80

$\bar{x} \pm 2\ \sigma$ = 44,6 - 91,8

V = 17,30 %

Calculating pertussis

σ = 2,02

$\bar{x} \pm 1\ \sigma$ = 6,78 - 10,82

$\bar{x} \pm 2\ \sigma$ = 4,76 - 12,84

V = 22,95 %

Conclusions:

-All the measured values show that the method has satisfying accuracy. Results from potency test are not different from expected and complete the requirements, according to European Pharmacopoeia and the specification for lower and upper limit of the products.

-Results show that the found values according to standard deviation σ are acceptable - all produced batches are limited in $\bar{x} \pm 2\ \sigma$ and the condition for precision is done. When obtained data values are in $\bar{x} \pm 3\ \sigma$ they are unacceptable – alert limit.

-Coefficient of variation (v) is from 13 % to 24%, which for biological tests with animals is a measure of good repeatability and specificity (WHO P2 Validation).

-Results do not change from different seasons or current performer, which indicates reliability during normal assay conditions.

-Calculations, made with "probit" analysis, do not show deviation from linearity and range. LD_{50} - values both for standard and tested vaccines are between maximum and minimum concentration.

-Doing the analyses of results, problems for validation are not found.

-All the batches meet requirements of European Pharmacopoea.

-The potency test gives reliable results and it may also be used for the same purpose by same condition of performance.

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ADSORPTION POWER OF *ALUMINIUM HYDROXIDE* USED AS ADJUVANT IN BACTERIAL VACCINES

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SUMMARY

Aluminium hydroxide is commonly used as an adjuvant in the production of bacterial vaccines. It serves as a carrier of the immunogen thus bringing it in the most effective form to the organism. The capacity of the aluminium hydroxide to accumulate the antigen is an important characteristic, which must be estimated in order to evaluate its adjuvant properties. The adsorption power of the aluminium hydroxide is a useful measure of its capacity. Therefore the adsorption of bovine serum albumin (BSA) on aluminium hydroxide produced in BB - NCIPD was studied. The relationship between concentration of BSA and percentage of adsorption on aluminium hydroxide was established. The adsorption capacity of the aluminium hydroxide produced at BB-NCIPD was characterized.

Key words: Aluminium hydroxide, adjuvant, vaccine, adsorption capacity, bovine serum albumin (BSA).

INTRODUCTION

Aluminium hydroxide and aluminium phosphate adjuvants are widely used in both human and animal vaccines (1). Aluminium hydroxide and aluminium phosphate along with calcium phosphate are for the time being the only adjuvants accepted by the European Pharmacopoeia (2) for use in human vaccines. Effective adjuvanticity depends on complete adsorption of the antigen to the aluminium salt (3) The European Pharmacopoeia and the World Health Organization allow up to 1.25 mg aluminium per dose of aluminium-containing vaccine.

The purpose of this work was to investigate the adsorption capacity of the BB-NCIPD produced aluminium hydroxide gel used as adjuvant for the bacterial vaccines. It was well established that the adsorption of proteins on aluminium gels was pH-dependent with maximum adsorption occurring under pH conditions where the antigen and adjuvant had opposite charge (4). The point of zero charge (pZC) and the isoelectric point (pI) are used to characterize the charge of aluminium-containing adjuvants and antigens, respectively (5). The net surface charge is positive when the pH is below the pZC or the pI. Likewise, the net surface charge is negative when the pH is above the pZC or the pI. At pH around 7.0 the strong adsorption of the negatively charged BSA (pI 4.8) by positively charged aluminium hydroxide (pZC 11.1) was observed (6). Conversely, when the protein and adjuvant had the same charge, relatively little adsorption occurred. This behavior clearly demonstrated that electrostatic forces contributed to adsorption.

ABBREVIATIONS USED IN THIS PAPER:

BSA – bovine serum albumin, Al – aluminium, A_{750nm} – absorption at 750 nm.

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MATERIALS AND METHODS

1. Bovine serum albumin (BSA), Sigma. Solutions of BSA in distilled water with concentration 0.1, 0.2, 0.4, 0.6, 1.0 and 2.0 mg/ml.
2. Aluminium hydroxide produced in BB - NCIPD – different lots.
3. Procedure.

3.1 Aluminium hydroxide was diluted with distilled water to an aluminium concentration of 1mg/ml.

3.2 For adsorption, 1 part of the diluted gel was mixed with 4 parts of each of the solutions of BSA and allowed to stand at room temperature for 1 h. During this time the mixture was shaken several times. Afterwards the samples were centrifuged and the protein content of the supernatants was determined immediately by the Lowry procedure (7).

RESULTS AND DISCUSSION

The results from the adsorption experiments with five different lots of aluminium hydroxide produced in BB-NCIPD at different times are given in table 1.

The supernatants after adsorption of solutions with 0.1 mg/ml, 0.2 mg/ml and 0.4 mg/ml concentration of BSA on the gel were practically free of BSA since the A_{750nm} was under 0.010, i.e. the residual protein left was around or below 5 μg/ml. Solutions with BSA concentration of 0.6 mg/ml and 1.0 mg/ml were also very well adsorbed on the gel. Concentrations of BSA of 2.0 mg/ml and above were already high and with them only partial adsorption of the protein on the gel occurred. The results show that the adsorption of protein proceeds in the same way with the different lots of aluminium hydroxide, since the residual protein in the supernatants was almost equal with all lots tested.

Table 1. Adsorption of bovine serum albumin (BSA) on aluminium hydroxide

Aluminium hydroxide, Lot No.	Supernatant after adsorption of BSA solutions with concentration,mg/ml	A_{750nm}	Result Residual BSA in the supernatant, μg/ml
279	0.1 mg/ml	0.005	3
	0.2 mg/ml	0.007	4
	0.4 mg/ml	0.010	5
	0.6 mg/ml	0.014	7.5
	1.0 mg/ml	0.016	8
	2.0 mg/ml	0.686	5>200
286	0.1 mg/ml	0.001	0
	0.2 mg/ml	0.007	4
	0.4 mg/ml	0.011	6
	0.6 mg/ml	0.014	7.5
	1.0 mg/ml	0.060	30
	2.0 mg/ml	0.829	30>200
293	0.1 mg/ml	0.005	3
	0.2 mg/ml	0.008	4
	l0.4 mg/ml	0.009	5
	0.6 mg/ml	0.011	6
	1.0 mg/ml	0.022	11
	2.0 mg/ml	0.734	>200
298	0.1 mg/ml	0.001	0
	0.2 mg/ml	0.006	3.5
	0.4 mg/ml	0.009	5
	0.6 mg/ml	0.009	5
	1.0 mg/ml	0.053	25.5
	2.0 mg/ml	0.851	>200
305	0.1 mg/ml	0.003	2
	0.2 mg/ml	0.008	4
	0.4 mg/ml	0.010	5.5
	0.6 mg/ml	0.015	8
	1.0 mg/ml	0.055	27
	2.0 mg/ml	0.768	>200

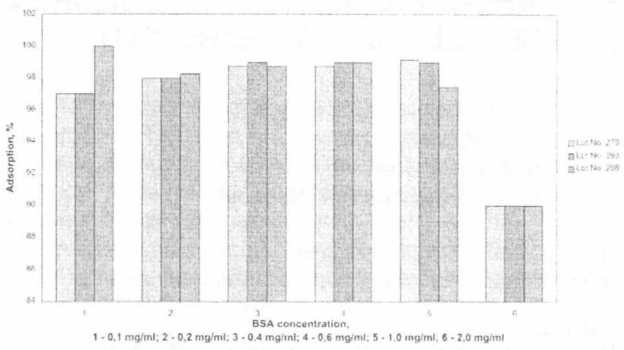
According to the requirements of the European Pharmacopoeia an aluminium hydroxide is suitable for use as an adjuvant in the production of vaccines if no BSA is found after adsorption in the supernatants with BSA concentration of 0.4 mg/ml and lower. As it can be seen from the table all lots confirm to this requirement since the residual BSA in all cases was negligible.

The aluminium hydroxide adsorbed 97% to 100% of the BSA from the solutions with concentration of up to 1.0 mg/ml that is shown on fig.1 for three of the tested lots. The lower adsorption power (97%) at the lowest BSA concentration used (0.1 mg/ml) for lot No. 279 may be explained with the insufficient amount of protein to saturate the surface places on the gel.

Taking into account all results the protein adsorption capacity of the aluminium hydroxide produced in BB – NCIPD was determined. It was calculated in mg protein/mg aluminium (Al) on the basis of the adsorption of the BSA solutions with 1.0 mg/ml concentrations. The adsorption capacity of all tested lots of aluminium hydroxide was of the same order, which is evident from the data on fig.1, column 5, and was estimated to be 3.9 mg/mg Al. Other authors have found an adsorption capacity of 2.9 and 2.7 mg/mg Al for two commercial aluminium hydroxide adjuvants (8). The higher adsorption capacity of our gels shows that they exhibit an increased surface area that is available for adsorption of protein. The adsorption capacity of the BB – NCIPD manufactured aluminium hydroxide is a constant characteristic of the product which allows the production of an aluminium adjuvant of very good adsorption power for use as an adsorbent in the bacterial vaccines.

At this time the only adjuvants currently licensed for human use are aluminium and calcium salts. These salts have been extensively characterized and are commonly used with various vaccines to increase the magnitude of humoral immune responses (9). This work is part of a study aimed to characterize the stability of the bacterial vaccines produced in BB – NCIPD. The results obtained clearly demonstrate the suitability of the aluminium hydroxide as an adjuvant for the bacterial vaccines. The strong contribution of electrostatic interactions to adsorption of BSA or other proteins on the aluminium hydroxide is connected with more likely elution upon intramuscular or subcutaneuos administration (10).

Fig. 1. Adsorption capacity of aluminium hydroxide



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PROTECTION AGAINST WHOOPING COUGH IN CHILDREN BETWEEN 0 – 6 YEARS OLD

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SUMMARY

The specific immunoprophylaxis of humans with pertussis, as a component of combine bacterial vaccine leads to production of specific antibodies that is indicator of the whooping cough prevention. For evolution of immunization procedures and the vaccine itself, antibody levels against pertussis are useful to show the immune status of the population. An enzyme-linked immunosorbent assay was used for measuring immunoglobolin G pertussis antibodies in human sera. The ELISA involves the blinding of bacterial cells to polystyrene tubes. Results of the direct ELISA test are highly reproducible. It is believed that a pertussis antibody level of 1:81 provides protection against disease. The titters of antibody from 1:161 to 1:320 showed full protection of people against whooping cough and titters over 1:321 are used as a criteria of disease or used as a criteria of passed disease. The results of estimation of immune status of population in Bulgaria were based on the same criteria.

The assay was done in plastic plates coated with inactivated bacterial cells. For investigation 279 human sera were tested in age group between 0 – 6. The tested sera showed that 54% of persons have full protection against whooping cough and in 42% of children the obtained titters showed basic immunity. In 4% of patients the titter of antibody is more than 1:321, which titter is used as a criteria of disease of human sera. All of sera have a level enough for protection against whooping cough and non-protected patients were not found.

Present results indicate a good protection against pertussis in Bulgaria in children till 6 years old. This fact is a result of specific immunoprophylaxis of infants up to 2 years of age by pertussis vaccine, as a component of DIFTETKOK (DTP) combined bacterial vaccine, produced by BB – NCIPD, Ltd., Sofia Bulgaria.

Key word: *pertussis, whooping cough, ELISA, immune status.*

INTORDUCTION

Whooping cough - or pertussis - is an infection of the respiratory system caused by the bacterium *Bordetella pertussis* (or *B. pertussis*) (1). It's characterized by severe coughing spells that end in a "whooping" sound when the person breathes in. Before a vaccine was available, pertussis killed 5,000 to 10,000 people in the United States each year.

Now, the pertussis vaccine has reduced the annual number of deaths to less than 30. But in recent years, the number of cases has started to rise. By 2004, the number of whooping cough cases spiked past 25,000, the highest level it's been since the 1950s (8). The morbidity rates of whooping cough in Bulgaria reduced from 12.90% 000 children in 1955 to 2.85% 000 in 2005. It's mainly affected infants who are younger than 6 months old before they are adequately protected by their immunizations, and kids who are 11 to 18 years old whose immunity has faded. Although whooping cough can occur at any age, it's most severe in unimmunized children and in infants under 1 year of age (early immunization can usually prevent this serious disease in babies) (2). But more cases have been reported in teens and adults, because their immunity has faded since their original vaccination.

If whooping cough is suggested as a diagnosis it is natural to ask how it can be proved or disproved. Unfortunately there is no easy way. The usual way is to try to detect the causative organism (Bordetella pertussis) in the back of the nose (12). This usually involves passing a swab on a wire through a nostril to the back of the throat and sending it to a medical lab to culture the material. This may take 5 to 7 days. If Bordetella pertussis grows this is usually taken as proof that it is whooping cough (8). Unfortunately the organisms is delicate, killed easily by many antibiotics and has often been eliminated from the body by natural defences by the time the diagnosis is suspected. It is easiest to find it in the first 2 weeks but very unlikely after 3 weeks. But the patient has often had it for 3 weeks before whooping cough is suspected. So it is unusual to get a positive culture in whooping cough. In other words, if a swab is negative, the patient can still have whooping cough (14).

A better and more modern way of detecting the disease is by an enzyme - linked immunosorbent assay (ELISA) that measures the level of antibody response against pertussis by detection of Ig G antibody (3, 9). The high sensitivity and specificity of the ELISA allowed for antibody determination in small amounts of sera for short time. Antibody tests are done by some laboratories on blood samples taken after several weeks of illness. By looking at IgG and IgA antibodies to fimbria, pertussis toxin and filamentous haemagglutinin, it is possible to say whether it is likely the patient has had whooping cough (6, 7).

Knowledge of the immune status of population against pertussis has big practicable implication. It may assist in checking the efficacy of immunization schedules adopted and the persistence of immunity. Therefore was necessary to make a screening for level of the protection against pertussis of the population in Bulgaria.

In this paper we present our results of estimation of the immune status of the children up to 6 years old by ELISA method for immunoglobolin G antibodies against whooping cough in human sera during the five years investigation period.

MATERIALS AND METHODS

Human sera.

The epidemiological study on pertussis immunity was carried out on 279 subjects, selected by Hygiene Epidemiological Inspection, Sofia, Bulgaria. Sera separated from clotted blood were stored at - 20° C in small vials and were tested for pertussis antibodies using ELISA method (15).

The obtained results were classified depend of the titer of antibody in following subgroups: subgroup of sera with the level of antibody up to 1:80 - group of non protective people; subgroup with level of antibodies against pertussis between 1:81 to 1:160 - sera with basic immunity, subgroup with level of antibodies against pertussis between 1:161 - 1:320 - sera with full protection and subgroup with level of antibodies against pertussis more than 1:321, human sera with titer used as a criteria for illness or past disease (5).

ELISA (3).

The BA test (13) is used for preparation of serum standard for detection of the titers by ELISA. This was the first method

ABBREVIATIONS USED IN THIS PAPER:
PBS - phosphate buffered saline; OPD - ortophenilenediamine.

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developed to measure pertussis antibody and it is still the most frequently used method. Bordetella pertussis suspension (serotype 1, 2 and 3), (batch 36, BB – NCIPD, Ltd. - Sofia, Bulgaria), dued and inactivated with 0.01% thiomersal was used in BA test. Diluting the stock suspensions to opacity 10 OUs made working dilutions. Sera samples were prepared in following concentrations: 1:10; 1:20; 1:40; 1:80; 1:160; 1:320; 1:640; 1:1280; 1: 2560. The level of antibody is measured after 24 hours incubation at 37° C and following incubation for 24 hours at room temperature. The last dilution of human sera with agglutination is a titer of antibody against pertussis.Polystyrene plates with 96 wells (Nunc immunoplates, Denmark) were coated with bacterial cells in working dilutions 10 OUs (batch 39, BB – NCIPD, Ltd. - Sofia, Bulgaria). To prepare the plates the bacterial cells were diluted in chloroform:ethanol (1:10)
(4). 100 ml volumes were added to the wells and left 2 hours at 37° C and overnight at room temperature. The following morning the plates were emptied and drained over filter paper. After being washed three times with PBST (Phosphate Buffered Saline pH 7.2 containing 0.05% Tween 20), 100 ml volumes blocking buffer - PBS containing 1% BSA (Bovine Serum Albumin) were added to the wells and incubated at 37° C for 1 hour (11).
100 ml volumes of sera dilutions (1:50) in PBST were added to the wells of sensitized plates, incubated at 37° C for 2 hours and washed three times in PBST. 100 ml volumes of peroxides anti - human immunoglobolin G (National Centre of Infectious and Parasitic Diseases - Sofia, Bulgaria) diluted in PBST (1:500) was added to each well, followed by incubation at 37° C for 60 minutes and washing three times of the plates. The plates were than left at room temperature with 100 ml volumes of a chromogen solution (10 mg of ortophenilenodiamine and 10 ml of 30% hydrogen peroxidase in 50 ml citrate - phosphate buffer pH 5.0). The reaction was stopped after 30 minutes with 100 ml of 1 N H_2SO_4. The plates were directly read at 405 nm in a MicroELISA Minireader Photometer (Bio - Tek) in optical densities. Standard curve with five points according to measured optical densities of used control serum samples, standartizated by BA method, was prepared each time (10).

RESULTS AND DISCUSSION

We have tested 279 human sera by ELISA for estimation of pertussis antibodies and the results are presented on fig No 1, 2, 3.
The tested sera, of group of children till six years of age, showed that 54% of persons have full protection against whooping cough. In this group was not found subgroups of non-protected people. This fact is a result of the specific immunoprophylaxis with combine vaccine DTP following the shame recommended by the Bulgarian immunization program. The children with protective titter are more than 42%. Un protected sera were not found. This results confirmed the efficacy of immunization schedules adopted and the persistence of immunity.
The antibody response in unvaccinated patients was with increased the IgG titers. The low percent of children with titer more than 1:321 in 4% of human sera demonstrated that disease has been effectively controlled by vaccine prophylaxis.
All of sera have good level of protection against whooping cough and non-protected patients were not found.
The ELISA involves the blinding of bacterial cells to polystyrene tubes. Results of the direct ELISA test are highly reproducible. It is believed that a pertussis antibody level of 1:81 provides protection against disease. The titters of antibody from 1:161 to 1:320 showed full protection of people against whooping cough and titters over 1:321 are used as a criteria of disease or used as a criteria of passed disease. The results of estimation of immune status of population in Bulgaria in this group of age were based on the same criteria.
Summarized results of serological studies for five years, presented on Table No.1, showed good protection against pertussis (more than 90%). The prevalence of pertussis antibody in the general population depends on the status of immunization against pertussis in childhood.
Previous pertussis vaccinations of patients with whooping cough may interfere with the antibody response to the natural disease. Granstorm et al. (7) found that the antibody response in unvaccinated people different in their vaccinated counterparts: unvaccinated children had an early increase in Ig M titters and late Ig G response (more than 1:131), whereas most vaccinated children and adults had a secondary type response with an early increase in Ig A and the level of Ig G antibody is until 1:320.
Populations in other countries that have also been studied in this way appear to be somewhat heterogeneous on the basis of these criteria. However, according to our opinion, in most cases the population can be considered comparable and from this comparison, the epidemiological situation observed in Bulgaria appears more favourable than that of some European countries and of the USA. In Sweden, for instance, high proportion of children had experienced pertussis infection by age 10, in Czechoslovakia the percent of sero-positively decreased from 81% in persons 15 to 19 years old to 16% in persons 30 to 34 years and in Poland the children to one year the proportion with titer 1:40 or higher was 60%, while the proportion with a titer 1:160 or higher was 29%. Only 7% of person aged 15 years in Poland had a titer of 1:160 or higher. In the United States in 1998 there were 7,405 reported cases. There were 5 reported deaths. In 1999 there were 7,288 cases. In 2000 there were 7,867. The provisional number of deaths for 2000 is 12. Only 82% of children in the US are fully immunized against pertussis (2000). In England and Wales about 3 deaths per year are recorded. In 2004 there were 504 notifications. Probably the number of cases occurring is probably 50 times greater. The pertussis vaccine is estimated to be 63% to 94% effective in the DPT. The 4% of children in Bulgaria with titer more than 1:321 demonstrated the efficacy of administrated vaccine in the country.
Despite a very high vaccination rate in the world, thousands of cases occur. WHO reported that a growing number of pertussis cases are occurring in vaccinated adults. Often adults and teenagers can have atypical whooping cough and only exhibit symptoms similar to a bad cold or flu (5). The undiagnosed adult and teenage carriers of whooping cough, most of who have been fully vaccinated, spread the disease to vulnerable newborn infants and young children.
The 46 years of experience with the application of the bacterial vaccine DTP in Bulgaria and the testing of the immune status of the population give grounds for the following conclusions: The bacterial vaccine Diftetkok, produced by BB-NCIPD, Ltd., has a high level of immunogenicity. The antibodies' level against pertussis in tested human sera is enough to protect people from these diseases. The bacterial vaccine is effective preparation with a good safety profile for active immunization and indicate a good prevention against whooping cough in Bulgaria.
This is a result of specific immunoprophylaxis with pertussis vaccine, as a component of combined bacterial vaccine DTP up to 2 years old children following the shame recommended by the Bulgarian immunization program.

Table 1. Estimation of the immune status of population against whooping cough in period 2001 - 2005.

Year		2001 - 2005			
	Number of human sera	% Full protection 1:161 – 1:320	% Basic immunity 1:81 – 1:160	% Non - protected Less than 1:80	% of children with titter more than 1:321
2001	18	53	43	0	4
2002	19	54	44	0	4
2003	103	51	45	0	4
2004	42	53	44	0	3
2005	97	55	41	0	3
Total	279	54	42	0	4

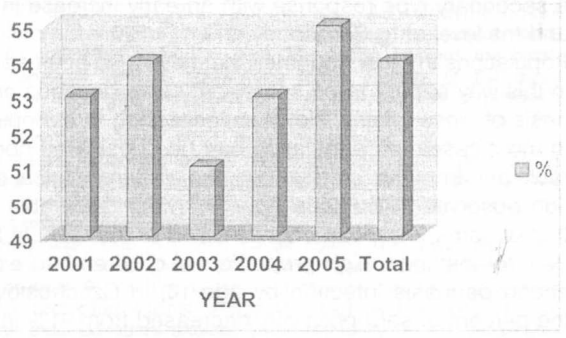

Fig. 1: Children with full protection against pertussis in period 2001 - 2005

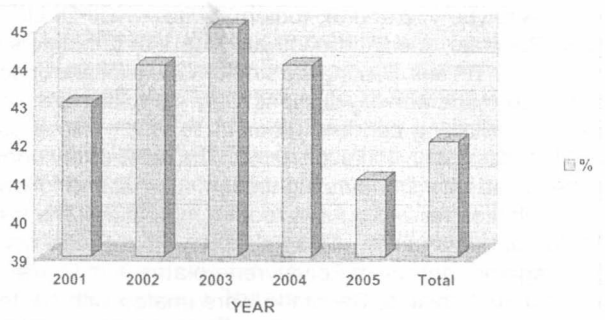

Fig. 2: Children with basic immunity against pertussis in period 2001 – 2005

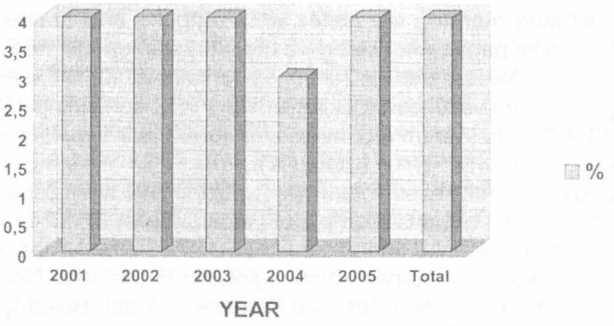

Fig. 3: Children with titter of antibodies against pertussis more than 1:321 in period 2001 – 2005

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STABILITY STUDIES OF BACTERIAL VACCINES PRODUCED IN "BUL BIO – NCIPD" LTD. TETATOX (TETANUS VACCINE ADSORBED) – ACCELERATED TEST

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Bul-Bio NCIPD

SUMMARY

To provide evidence on how the quality of the bacterial vaccines varies under the influence of environmental factors such as temperature and humidity, and to conform established shelf-life for the products in different dosage forms and confirm the recommended storage conditions accelerated tests were performed. The accelerated testing covers the attributes that are likely to influence the quality, safety and/or efficacy of the bacterial vaccines. The analytical procedures for the parameters are validated. The storage conditions and the lengths of studies chosen are sufficient to cover storage, shipment and subsequent use. The stability studies are performed with vaccines filled in different dosage forms (vials – 10 and 20 doses) with rubber stoppers and aluminum caps. The testing of the parameters is performed by approved methods at the Laboratory for Control of Human Medicines.

Key words: tetanus vaccine adsorbed, accelerated stability

INTRODUCTION

The vaccine Tetatox, produced in "Bul Bio – NCIPD" Ltd. is in use for many years. According the World Health Organization recommendations all manufacturers to perform stability studies including all dosage forms.

The purpose of the stability studies is to establish, based on testing a minimum of two or three batches of the finished product, a shelf life and label storage instructions applicable to all future batches of the finished product manufactured and packaged under similar circumstances. The degree of variability of individual batches affects the confidence that a future production batch will remain within specification throughout its shelf life.

Shelf-life acceptance criteria are derived from the collected stability information.

Significant changes are defined as:

- Failure to meet the criterion for potency of a product
- A 5% change in an assay from its initialize value for contents of adjuvant, sodium chloride, thiomersal, free formaldehyde
- Failure to meet the criteria for appearance and physical attributes for the suspension

Criteria to be tested according Specification:	
Appearance	Homogenised product consisting of a turbid, whitish to light beige suspension
pH	6,0 - 7,0
Adjuvant	as Al^{+++} $\leq$ 1,25 mg/dose
Sodium Chloride	7-10 g/l
Thiomersal	$\leq$ 0,05 mg/dose
Free formaldehyde	$\leq$ 0,2 g/l
Potency **T** - IU/dose	$\geq$ 40 IU/dose
Sterility	sterile

- Failure to meet the acceptance criterion for pH

If no significant change occurs at the accelerated condition, the retest period and the confirmation of the established shelf-life depend on the nature of the long-term and accelerated data.

For the purpose of the report to WHO, if no significant change is found at the accelerated condition, extrapolation will be made for the whole shelf-life of the product and the company will be committed to perform long-term tests.

If significant change is found between 3 and 6 months at accelerated storage condition the proposed and already established shelf-life will be based on long term data.

VVM reaction rates by category of heat stability and temperature and time period for testing

CATEGORY (Vaccines)	N days to end point at +37°C	N days to end point at +25°C	Time to end point at +5°C
VVM 30			
HIGH STABILITY	**30**	**193**	**> 4 years**
VVM 14			
MEDIUM STABILITY	**14**	**90**	**> 3 years**
VVM 7			
MODERATE STABILITY	**7**	**45**	**> 2 years**
VVM2			
LEAST STABILITY	**2**	**NA***	**225 days**

* - VVM *(Arrhenius)* reaction rates determined at 5 and 37 degrees C. Unless otherwise specified, the two temperatures and time periods highlighted in Table will be the agreed test period for testing each VVM category. Additionally, each VVM category is tested at time greater than the end point time to verify that all VVM samples will reach the end point.

In the accelerated test two different lots of Tetatox are included. Each of them includes two different sublots with dosage form of 5 ml (10 human doses) and 10 ml (20 human doses) packaged in vials I hydrolytic class, total volume 10 ml and packed in carton boxes x 10.

One sublot of each lot is filled into vials (5 ml and 10 ml) with rubber stoppers and aluminum caps for testing the stability of the product. After the testing of the dosage forms is performed, certificate of analysis is issued (time 0). The products are packed in carton boxes and the packaging proposed for storage and distribution is simulated.

The vaccine samples has been kept exposed to +37$\pm$0,2 °C and with relative humidity of 33% $\pm$ 5% in a temperature-controlled cabinet without light.

The raw data of the performed tests and obtained results are filled in the Master Form for stability testing. A tabulated summary is prepared for each testing period and for each dosage form is prepared.

Row data are composed graphically to upper and lower acceptance criteria over the time periods; upper and lower confidence limits are determined were applicable.

Seventeen vials of each sublot of Tetatox vaccine were placed in a temperature-controlled cabinet without light. The vaccine samples has been exposed to +37 $\pm$ 0,2°C with relative humidity of 33% $\pm$ 5%in for 14 days. At the end

ABBREVIATIONS USED IN THIS PAPER: NONE

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of this period the test for determination of the potency has started (immunization of the animals). The other tests for determination of pH, aluminium content (adjuvant), determination of sodium chloride, determination of thiomersal, determination of free formaldehyde are performed. The test for sterility is started too (inoculation).

MATERIALS AND METHODS

All the methods of testing are approved by Bulgarian Drug Agency (BDA).

Appearance

Method

The adherent labels are removed from the containers and the containers are washed and dried the outside surface. The container is gently swirled or inverted, ensuring that air bubbles are not introduced, and observed for about 5 seconds in front of the white panel. The preparation fails the test if one or more particles are found in more than one container.

Apparatus

The apparatus consists of a viewing station comprising of:
- matt black panel of appropriate size held in a vertical position
- a non-glare white panel of appropriate size held in a vertical position next to the black panel
- an adjustable lamp holder fitted with a suitable shaded, white - light source and with a suitable light diffuser. The intensity of illumination at the viewing point is maintained between 2000 and 3750 lux although higher values should be used for colored glass.

Determination of pH

Method

The potentiometric determination of pH is based on the comparison of the potential of the indicator electrode immersed in the examined solution with the potential of the same electrode immersed in a standard buffer solution with known pH.

Equipment, Reagents and solutions

Standard buffer solution with pH 4
Standard buffer solution with pH 7
pH - meter type 64 Radiometer

The pH is measured twice (using two samples) and the mean value is calculated.

Aluminium content

Method

Aluminium content is determined by complexometric titration.

To 1 ml of the sample placed in a conical flask 1 ml of sulfuric acid and 0.1ml of nitric acid ere added. The solution is heated until the dissolution of the aluminium hydroxide. After cooling, 10 ml of water for injections (WFI) and 0.05 ml of methyl orange solution are added. Neutralisation with sodium hydroxide solution follows (the colour changes to yellow). After that 25 ml of WFI, 10 ml of 0.01 mol/l sodium edetate and 5 ml of acetate buffer are added. The mixture is heated for 5 min on a water bath, 0.5 ml of the indicator pyridylazonaphthol are added and a titration is performed with 0.01 mol/l copper sulphate solution until the colour changes to purplish-violet. A blank titration replacing the sample with WFI is carried out. The aluminium content in the sample is calculated according to the following equation:

$$\text{Aluminium, mg/ml} = \frac{Va - Vb}{C} \times 0.2698$$

where,

Va – copper sulphate required to titrate the blank in ml
Vb – copper sulphate required to titrate the sample in ml
C – volume of the sample in ml
0.2698 – quantity of aluminium in mg equivalent to 1 ml of 0.01 mol/l sodium edetate

Equipment, Reagents and solutions

Sulfuric acid, conc. 95-97%
Nitric acid, conc. 65%
Methyl orange solution
Sodium hydroxide solution, 500 g/l
Sodium edetate, 0.01 mol/l, Titrisol
Acetate buffer, pH 4.40
Pyridylazonaphthol solution
Copper sulphate, 0.01 mol/
Analytical balance "Sartorius"
pH – meter type 64 "Radiometer"

The Aluminium content is measured twice (using two samples) and the mean value is calculated.

Determination of sodium chloride

Method

Sodium chloride determination is performed by automatic potentiometric titration. The end-point of the titration is determined by the change of the potential difference between two electrodes (one indicator electrode and one reference electrode), immersed in the solution to be examined, as a function of the quantity of titrant added.

To 0.2 ml of the sample 50 ml of water for injections (WFI) and 4 ml of 2 mol/l nitric acid are added. The solution is titrated with 0.1 mol/l silver nitrate solution on an automatic titration instrument type 720 KFS Titrino. The result is expressed in g/l.

Equipment, Reagents and solutions

Nitric acid, conc. 65%
Nitric acid, 2 mol/l
Silver nitrate, 0.1 mol/l, Titrisol
Titration instrument type 720 KFS

The Sodium chloride content is measured twice (using two samples) and the mean value is calculated.

Determination of thiomersal

Method

The method for determination of thiomersal is based on the change of mercury from thiomersal to ionic state followed by colorimetric measurement of mercury dithizonate.

To 0.2 ml of the examined sample 1.2 ml of diluted sulfuric acid is added.

The test flask is heated on a water bath to dissolve the aluminium hydroxide in adsorbed with aluminium hydroxide gel vaccines. After cooling 5.0 ml of potassium permanganate solution are added and the solution is left at room temperature for one hour. 1.5 ml of hydroxylamine sulfate solution, 35.0 ml of water for injections, 5.0 ml of acetic acid solution are added and the mixture is shaked vigorously. To this solution 10.0 ml of dithizone solution in chloroform are added and the mixture is shaked vigorously for 30 sec. After that the content of each sample is transferred to a separation funnel. The absorbance at 490 nm of the separated lower chloroform layer is measured against the blank containing water for injections instead of the sample. The volume of mercury in the sample, read from the standard curve, is automatically shown on the display of the spectrophotometer.

The thiomersal content is calculated according to the following equation:

$$\text{Thiomersal, mg/ml} = \frac{V.A.100}{0.2.49.55}$$

where:

V - the volume (ml) of the working Hg standard, measured from the standard curve, containing equal quantity of Hg as that in the sample.

A - Hg content in the working standard (mg / ml)
0.2 - the sample content (ml)
49.55 - Hg content in 100 parts of thiomersal
Equipment, Reagents and solutions
Sulfuric acid, conc. 95-97%
Sulfuric acid / water for injections (V/V=1/1)
Potassium permanganate solution, 50 g/l
Glacial acetic acid solution, 6 mol/l
Hydroxylamine sulfate solution, 200 g/l
Chloroform
Dithizone
Dithizone solution in chloroform, 0.01 g/l
Spectrophotometer Secomam type S750 I
The Thiomersal content is measured twice (using two samples) and the mean value is calculated.

Determination of free formaldehyde

Method
Acetylacetone reacts in a weak acidic solution with formaldehyde forming a yellow condensation product.
A 1 in 10 dilution of the vaccine is prepared to be examined. To 1 ml of the dilution 4 ml of water for injections and 5 ml of acetylacetone reagent are added and mixed well. The tube is placed in a water-bath at 40°C for 40 min. the tubes are examined down their vertical axes. The solution is not more intensely coloured than a standard prepared at the same time and in the same manner using 1 ml of formaldehyde standard solution containing 20 mg/l formaldehyde instead of the dilution of the vaccine to be examined.
Equipment, Reagents and solutions
Formaldehyde solution (35 per cent)
Formaldehyde standard solution (20 mg/l)
Ammonium acetate solution (150 g/l)
Acetylacetone
Glacial acetic acid
Acetylacetone reagent
Analytical balance Sartorius
Water bath
The Free formaldehyde content is measured twice (using two samples) and the mean value is calculated.

Potency test

The tests are performed under the recommendations of the European Pharmacopoea 5.0
Method
The potency of the vaccine is determined by comparison with National Standard calibrated against the International Standard foe Tetanus Toxoid, Adsorbed - inoculation of guinea-pigs with appropriate doses/dilutions of both the tested product and the reference material. A four-dilution assay is used. The potency of tetanus vaccine (adsorbed) is determined by comparing the dose of the vaccine required to protect guinea-pigs from the effects of a subcutaneous injection of a lethal dose of tetanus toxin with the dose of a reference preparation, calibrated in International Unit, needed to give the same protection.
The procedure is applied for the control of the final bulk (according to the specification of final bulk) and for the determination of the potency during stability studies.
Selection and distribution of the test animals
In the test healthy guinea-pigs each weighing 250-350g are used. The guinea-pigs are distributed in four equal groups. Each group containing eight animals. Four further groups of five quinea-pigs are included as anvaccinated controls. Guinea-pigs of the same sex are used in different groups or with the males and females equally distributed between the groups.
Preparation of the challence toxin solution.
Immediately before use, the challenge toxine is diluted to solution containing approximately 50 LD_{50} per ml. Dilute portions of the challenge toxin solution 1 in 16, 1 in 50 and 1 in 160 with the same diluent.

Determination of the potency of the vaccine

Using a 9 g/l solution of sodium chloride R, dilutions of the vaccine to be examined and of the reference are prepared, such that for each, the dilutions form a series differing by not more than 2,5 fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 1,0 ml per guinea pig, protect approximately 50 per cent of the animals from the lethal effects of the subcutaneous injection of the quantity of tetanus toxin. One ml of each dilution is injected subcutaneously into each guinea-pig. After 28 days, inject subcutaneously into each animal 1,0 ml of the challenge toxin solution (50 LD_{50}).
Twenty eight days after the immunization animals are injected with 1 ml of tetanus toxin, containing 50 LD_{50}. The injection is made mid-ventrally directly behind the sternum with the needle pointing towards the neck of the guinea-pig.
The next table gives typical sing of tetanus and the way of reading the results (degree of paralysis).

Interpretation of results

The number of surviving guinea-pigs is counted 5 days after injection of the challenge toxin. The potency of the vaccine is calculated on the basis of the proportion of animals surviving in each of the groups of vaccinated guinea-pigs, using Probit analysis.

Sings according the European Pharmacopoea 5.0	Sings used in BB NCIPD	Meaning/observation/
Healthy animal	0	Healthy animal
T1	±	Slight stiffness of 1 forelimb, but difficult to observe
T2	+	Paresis of 1 forelimb which still can function
T3	++	Paralysis of 1 forelimb. The animal moves reluctantly, the body is often slightly banana-shaped owing to scoliosis
T4	+++	The forelimb is completely stiff and the toes are immovable. The muscular contraction of the forelimb is very pro nounced and usually scoliosis is observed.
T5	#	Tetanus seizures, continuous tonic spasm of muscles
death	⊞	***death***

Requirements for a valid assay
The test is not valid unless:
- test vaccine and reference preparation 50 per cent protective dose lies between the largest and smallest doses of the preparations given to the quinea pigs
- the number of animals that die in the four groups of five injected with the challenge toxin solution and its dilutions indicates that the challenge toxin is approximately 50 LD_{50}.
- the fiducial limits of the assay (p= 0.95) fall between 50 per cent and 200 per cent of the estimated potency
- the statistical analysis shows no deviation from linearity and parallelism
The test may be repeated but when more than one test is performed the results of all valid tests must be combined in the estimate of potency.

Sterility test

Materials and Equipment
A/ Clean premises - the test is performed in laminar flow

hood class A, situated in clean room class B, according to the GMP rules.
B/ Pippettes: sterile pipets of 25 ml - Mor, pipets of 1 ml,2 ml; 5 ml and automatic pippets "Pipetboy".
C/ 22,5 ± 2,5°C Incubator and 32,5±2,5 C Incubator
D/ Culture media
Fluid thioglycollate medium
Soya-bean casein digest medium
The culture media is tested for Sterility and Growth promotion:
Direct inoculation of the culture medium (test for sterility)
The quantity of the preparation is transfer directly into the culture medium so that the volume of the product is not more than 10 per cent of the volume of the medium.
Final product
- Each ampoule/vial of adsorbed vaccine is transferred in 1 test-tube thioglycollate and soya-bean digest medium(with neutralizing substance).
- 2 test-tubes of sodium chloride (9 g /l) are put in one thioglycollate and soya-bean digest medium (with neutralizing substance) /negative control/.
- The test-tubes (x20 for each medium) with thioglycollate medium, in which the inoculation is made, are incubated for 14 days at 32,5 ± 2,5°C and soya-bean digest medium 22,5 ± 2,5°C
- 14 days after the beginning of incubation transfer portions
- (x1 ml) of the medium to fresh tubes of the same medium and then incubate the original and transfer tubes for 7 days
- Each one of the negative controls is cultivated at the temperatures shown above for 21 days.

Quantity per container	Minimum quantity to be used for each medium unless otherwise justified and authorised
Liquids	
- less than 1 ml	The whole content of each container
- 1-40 ml	Half the contents of each container, but not less than 1 ml
- greater than 40 ml and not greater than100 ml	20 ml
- greater than 100 ml	10 per cent of the contents of the container but not less than 20 ml

Number of items in the batch	Minimum number of items to be tested for each medium, unless otherwise justified and authorised
Parenteral preparations	
- not more than 100 containers	10 per cent or 4 containers, whichever is the greater
- more than 100 but not more than 500 containers	10 containers
- more than 500 containers	2 percent or 20 containers whichever is less

OBSERVATION AND INTERPRETATION OF RESULTS

At intervals during the incubation period and at its conclusion, examine the media for macroscopic evidence of microbial growth. If no evidence of growth is found the product to be examined complies with the test for sterility. If evidence of microbial growth is found the product to be examined does not comply with the test for sterility, unless it can be demonstrated by retest or by other means that the test was invalid for causes unrelated to the product to be examined. The test may be considered invalid only if one or more of the following conditions are fulfilled:
a) the data of the microbiological monitoring of the sterility testing facility show a fault
b) a review of the testing procedure used during the test in question reveals a fault
c) microbial growth is found in the negative controls
d) after determination of the identity of the microorganisms isolated from the test, the growth of this/these species may be ascribed unequivocally to faults with respect to the material and technique used in conducting the sterility test procedure. If the test is declared to be invalid it is repeated with the same number of units as in the original test.
If microbial growth is found in the repeat test the product examined does not comply with the test for sterility

RESULTS

Evaluation of stability data - p. 39-40

CONCLUSIONS

In the tested periods, with different storage conditions the chemical and biological performance of Tetatox vaccine stays in the limits of specification. It is necessary to notice that the potency of the vaccine is not reduced under critical lower limit.
The results are used to confirm appropriate storage conditions and expiry date.
Shelf-life acceptance criteria are derived from the collected stability information.
No significant changes are find:
- The criterion for potency of a product is higher than the lower limit and satisfies the requirements.
- There are no significant changes (5% or more from its initialize value) in an assays for contents of adjuvant, sodium chloride, thiomersal and free formaldehyde
- There is no failure to meet the criteria for appearance and physical attributes for the suspension
- The results of testing for pH are between the limits and there are no significant changes from its initialize value
No significant change is found at the accelerated condition.
The results of the accelerated tests at 37°C for 14 days and 25°C for 90 days are satisfactory and the VVM14 are suitable for the vaccines.

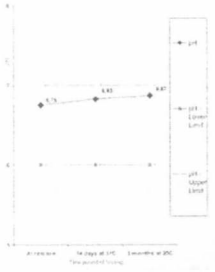
REFERENCES:
1. The European Agency for the Evaluation of Medicinal products (EMEA) – "Note for guidance on evaluation of stability data" (CPMP/ICH/420/02) – February, 2003
2. The European Agency for the Evaluation of Medicinal products (EMEA) Evaluation of Medicines for Human Use – "Guideline on stability testing: stability testing of existing active substances and related finished products) – December, 2003
3. "Procedure for assessing the acceptability, in principle, of vaccines for purchase by United Nations agencies" WHO, October, 2004

BUL BIO-NCIPD Ltd.	QC DepartmentStability of TETATOX - Summary of Results (Accelerated Test)				
Container Closure System	Stability is conducted on the dosage from packaged in vials I hydrolytic class, total volume 10 ml and packed in carton boxes x 1	Storage Conditions	From 2°C to 8°C Stored in a refrigerator	Do Not Freeze	Number of samples 240 vials

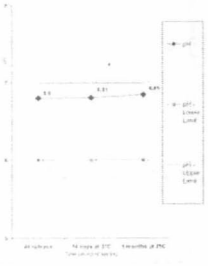
Lot N	Dosage form	Vial - 5,0 ml /10 doses/	Results	Storage at / time period / date	
187-12	Expiry date	10.2006	at	37+/-2°C	25+/-2°C / 60% RH +/-5%
	Batch size	240 vials	release	14 days / 12.2004	3 months / 02.2005
C r i t e r i a according Specification					
Appearance		Homogenised product consisting of a turbid, whitish to light beige suspension	Pass	Pass	Pass
pH		6,0 - 7,0	6,75	6,83	6,87
Adjuvant		as Al^{+++} ≤ 1,25 mg/dose	0,51	0,50	0,50
Sodium Chloride		7-10 g/l	8,0	8,2	8,2
Thiomersal		≤ 0,05 mg/dose	0,042	0,041	0,040
Free formaldehyde		≤ 0,2 g/l	< 0,2	< 0,2	< 0,2
Potency	T - IU/dose	≥ 40 IU/dose	* 88,95	82,15	80,16
Sterility		sterile	Pass	Pass	Pass

Lot N	Dosage form	Vial - 10,0 ml /20 doses/	Results	Storage at / time period / date	
187-12	Expiry date	10.2006	at	37+-2°C	25+-2°C / 60% RH +-5%
	Batch size	240 vials	release	14 days / 12.2004	3 months / 02.2005
C r i t e r i a according Specification					
Appearance		Homogenised product consisting of a turbid, whitish to light beige suspension		Pass	Pass
Pass					
pH		6,0 - 7,0	6,80	6,81	6,85
Adjuvant		as Al^{+++} ≤ 1,25 mg/dose	0,51	0,50	0,50
Sodium Chloride		7-10 g/l	8,0	8,2	8,0
Thiomersal		≤ 0,05 mg/dose	0,041	0,041	0,041
Free formaldehyde		≤ 0,2 g/l	< 0,2	< 0,2	< 0,2
Potency	T-IU/dose	≥ 40 IU/dos	* 88,95	79,90	85,32
Sterility		sterile	Pass	Pass	Pass

Stability - Accelerated Studies - pH of TETATOX

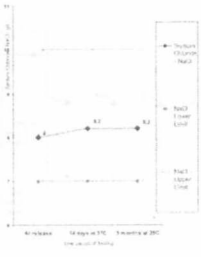


Lot N 187-12 / 5 ml



Lot N 187-12 / 12 ml

Stability - Accelerated Studies - Sodium



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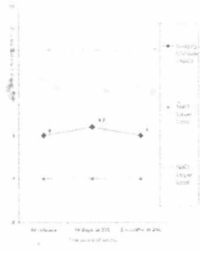
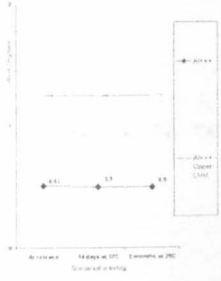


Lot N 187-12 / 12 ml

Stability - Accelerated Studies - Adjuvant /Al^{+++}/mg/dose



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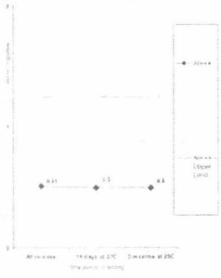
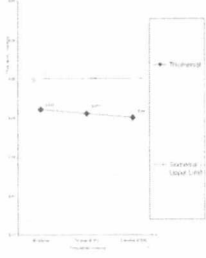
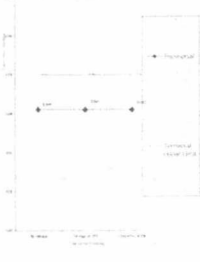


Lot N 187-12 / 12 ml

Stability - Accelerated Studies - Thiomersal - mg/

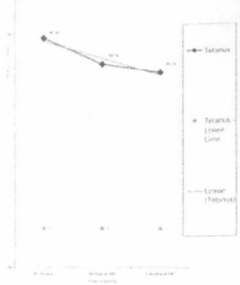


Lot N 187-12 / 5 ml

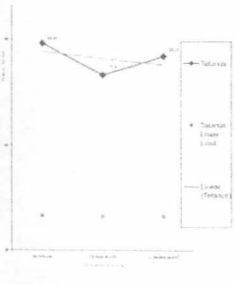


Lot N 187-12 / 12 ml

Stability - Accelerated Studies - Potency of TETATOX



Lot № 187-12 / 5 ml

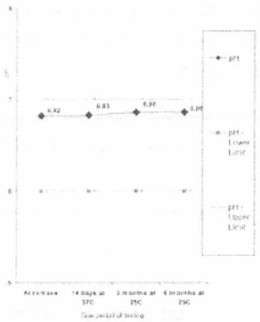


Lot № 187-12 / 12 ml

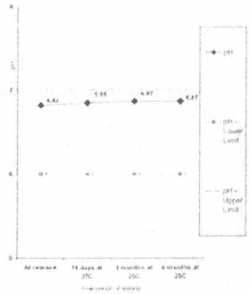
Lot N	Dosage form	Vial - 5,0 ml /10 doses/	Results	Storage at / time period / date		
A2188-12	Expiry date	10.2007	at	37+-2°C	25+-2°C / 60% RH +-5%	
	Batch size	240 vials	release	14 days / 12.2004	3 months / 02.2005	6 months / 05.2005
C r i t e r i a according Specification						
Appearance		Homogenised product consisting of a turbid, whitish to light beige suspension	Pass	Pass	Pass	Pass
pH		6,0 - 7,0	6,82	6,83	6,86	6,86
Adjuvant		as Al^{+++} J 1,25 mg/dose	0,55	0,56	0,55	0,56
Sodium Chloride		7-10 g/l	8,2	8,4	8,2	8,2
Thiomersal		≤ 0,05 mg/dose	0,042	0,042	0,041	0,041
Free formaldehyde		≤ 0,2 g/l	< 0,2	< 0,2	< 0,2	< 0,2
Potency T - IU/dose		≤ 40 IU/dose	* 94,54	73,79	87,12	86,66
Sterility		Sterile	Pass	Pass	Pass	Pass

Lot N	Dosage form	Vial - 10,0 ml /20 doses/	Results	Storage at / time period / date		
A3188-12	Expiry date	10.2007	at	37+/-2°C	25+2 °C / 60% RH +/-5%	
	Batch size	240 vials	release	14 days / 12.2004	3 months / 02.2005	6 months / 05.2005
C r i t e r i a according Specification						
Appearance		Homogenised product consisting of a turbid, whitish to light beige suspension	Pass	Pass	Pass	Pass
pH		6,0 - 7,0	6,82	6,85	6,87	6,87
Adjuvant		as Al^{+++} ≤ 1,25 mg/dose	0,56	0,56	0,55	0,55
Sodium Chloride		7-10 g/l	8,2	8,4	8,2	8,2
Thiomersal		≤0,05 mg/dose	0,042	0,041	0,041	0,041
Free formaldehyde		≤ 0,2 g/l	< 0,2	< 0,2	< 0,2	< 0,2
Potency T - IU/dose		≥ 40 IU/dose	* 94,54	72,95	80,00	82,15
Sterility		sterile	Pass	Pass	Pass	Pass

Stability - Accelerated Studies - pH of TETATOX

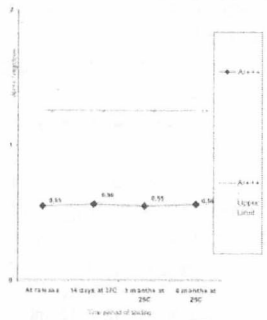


Lot N A2188-12



Lot N A3188-12

Stability - Accelerated Studies - Adjuvant /Al^{+++}/mg/dose



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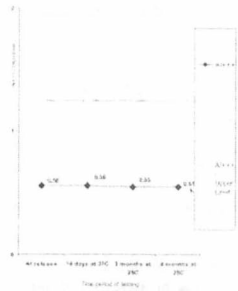
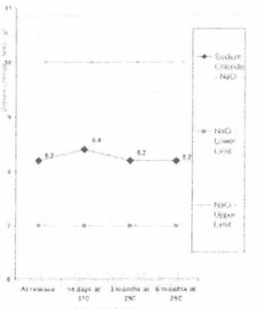


Lot N A3188-12

Stability - Accelerated Studies - Sodium Chloride-g/l of



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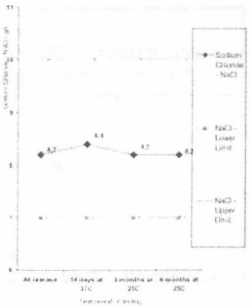
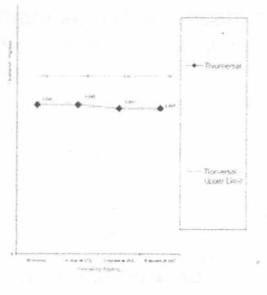
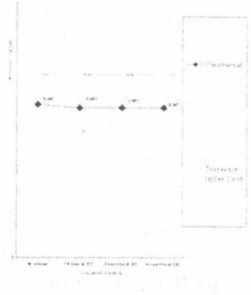


Lot N A3188-12

Stability - Accelerated Studies - Thiomersal - mg/dose of

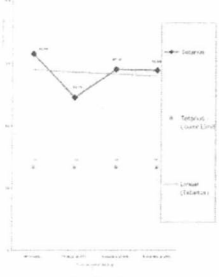


Lot N A2188-12

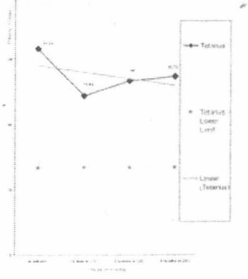


Lot N A3188-12

Stability - Accelerated Studies - Potency of



Lot N A2188-12



Lot N A3188-12