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IMMUNODIAGNOSIS OF HUMAN LISTERIOSIS IN PREGNANCY

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ABSTRACT

The recent increase in the incidence of the facultative bacterium *Listeria monocytogenes* severe infections of human and animals, makes it of significant public health importance . A total of 26 isolates of *L. monocytogenes* (23 from 23 patient group and 3 from 20 controls) were obtained from pregnant or non- pregnant women with spontaneous abortion and were submitted to serotyping . Only 2 *L. monocytogenes* serovars (1/2 a and 4b) are isolated in 90% of cases of human listeriosis . Of pregnant women sera , 56% were *L. monocytogenes* O and H agglutinating antibodies - positive while 100% and 78% of the cerebrospinal fluid (CSF) were *L. monocytogenes* O and H agglutinating antibodies positive, respectively . In the post-abortive subgroup , 100% and 90% were sero - and CSF -O and H agglutinins - positive , respectively.

The detection of anti-listeriolysin O (Anti-LLO) antibodies were done by Indirect Haemagglutination Reaction (IHR) with purified LLO in sera and CSF supernatant of 23 married women with frequent abortion due to *L. monocytogenes* infection and 20 controls to diagnose human listeriosis . The 23 patients (100%) with listeriosis and 3 (15%) of controls (non healthy subgroup), produced specific Anti-LLO titres ranged from < 100 to > 800.

INTRODUCTION

Listeria monocytogenes is a facultative, non sporulating, Gram positive intracellular bacterium widespread in nature and responsible for human and animal listeriosis [1]. *L. monocytogenes* causes a wide range of severe opportunistic infectious diseases in man; i.e. abortions, meningoencephalitis, septicaemia, pneumonia, endocarditis, localized abscesses, cutaneous lesions, conjunctivitis, urethritis, an infectious mononucleosis - like syndrome, hepatitis and arthritis [2-4] . Pregnant women, newborn babies , people over 40 years old and immunocompromised patients are at the greatest risk of getting listeriosis [3,5,6]. Listeriosis is the most commonly an intrauterine infection frequently as meningitis .

A pregnant women who develops listeriosis may have a low grade septicaemia. Trans-placental infection of the fetus may result in premature delivery of a stillborn or acutely ill infant with disseminated abscesses in the viscera (granulomatosis infantiseptica) and in the placenta [3,5,7,8] .

According to previous studies [9-11], the virulence of *L. monocytogenes* depends on its ability to synthesize at least two proteins: a hemolytic listeriolysin and a phosphatidylinostol - specific phospholipase-C. It seems certain , moreover, that listeriolysin is an intrinsic virulence factor that enables *L. monocytogenes* to escape from the phagocytic vacule of host cells and consequently to grow intracytoplasmically [12,13] and that this is an absolute requirement for the organism to cause infection. Listeriosis also seems to be needed by *L. monocytogenes* to infect neighbouring host cells at points of plasma membrane contact.

Virtually all pathogenic strains of listeria isolated from natural infections produce zones of β- hemolysis on blood agar medium [14-16] owing to production and release of one major listeriolysin from cells. Listeriolysin was successfully isolated from culture supernatents of certain strains of *L. monocytogenes* and shown to be a sulfhydryl- activated toxin sharing properties to other proteins (e.g., pneumolysin streptolysin O and perfringolysin) [1,17-20].

Fortunately the erythrocyte hemolytic activity of the purified Listeriolysin-O (LLO) had the lowest optimum pH (5.5) as compared to those of pneumolysin (pH 6.0), perfringolysin O and alveolysin (pH 6.5) and streptolysin-O (SLO) (pH 7.0) , hemolysins belong to *Streptococcus pneumoniae, Clostridium perferingens, Bacillus alvei* and hemolytic *streptococci*, respectively. In contrast to the other four hemolysins, the listerial hemolysin exhibited a narrow pH range of activity and hemolysis was not observed at pH 7.0 whereas the other toxins were mostly active at pH ≥ 6.50 to 7.0 [1].

The hemolysin (LLO) was apparently a heat - labile antigenic protein and its lytic activity was enhanced by reducing agents but suppressed by oxidation, cholesterol or to anti-SLO [1,17,21] . Rabbit antisera distinguishes 2 types of the hemolysin : α-listeriolysin and β-listeriolysin [20] . Recently, a number of rapid methods based on immunological or nucleic acid based procedures have been developed that aid in the rapid detection of the organism [22-27] . However, many of these lack sensitivity or specificity and no method allows for specific detection of both *L. monocytogenes* and other species .

This study was done to investigate whether detection of the specific anti-LLO , could be used for immunodiagnosis of human listeriosis or not . To date,

the functions of listeria virulence factors have only been demonstrated in vitro .The purpose of the present study was to examine and benefit in diagnosis, how virulence factors function in vivo to allow *L. monocytogenes* to establish infection, in the face of efficient early host defenses . This study presents a novel approach method of LLO indirect hemagglutination reaction (LLO-IHR) for immunodiagnosis of listeriosis in body fluids.

PATIENTS AND METHODS

Patients :

A group of 23 married - female - patients with frequent abortion due to severe listeriosis were studied. These patients were previously diagnosed as listeriosis depending on clinical and laboratory investigations and were under care in fevers Diseases Hospital, Zagazig and Alexandria , Egypt, during 1997-1998 . This was divided into 2 subgroups: 9 pregnant women with bacteraemia and 14 post abortive non pregnants, both aged from 33 to 40 years. The diagnosis of listeriosis (septicaemia and / or meningoencephalitis) was confirmed by isolation of *L. monocytogenes* from blood and cerebrospinal or placental fluids (CSF) . Bacterial cultivations (Blood or CSF samples) were grown or Brain Heart Infusion agar with 5% horse blood (blood agar) with / without nalidixic acid (1μg/ml).

Listeria monocytogenes :

Identification was established by conventional methods based on the usual criteria [28,29] . In addition API (RAPID) Coryne - System (API bio-Merieux, La Balme - Les- grottes , France) was also used [30-33]. Serotyping study was performed with the isolated *L. monocytogenes* and based on the agglutination reactions of listeria with highly absorbed rabbit antisera (Behring , Germany) to identify O (14 carbohydrate - containing heat stable) and H (5 heat - labile flagellar) antigens according to serotyping scheme of **Seeliger and Hohne** [35] and **Gellin** and his associates [34] .

Two control groups were assessed : 10 healthy married women without any complains and 10 married female patients with various active infections due to *Streptococcus pneumoniae* ; *Strephylococcus aureus* ; *Pseudomonas aeruginosa* ; *Escherichia coli* ; *Brucella abortus* or *Candida albicans* .

Methods :

Listeria agglutinating antibodies :

Bacterial O and H suspensions (serovars 1/2 and 4b, commercially purchased from Behring, Marburg, Federal Republic of Germany) were used as described by the manufacturer to detect serum and CSF agglutinating antibodies against *L. monocytogenes* O and H antigens . Doubling dilutions of sera or CSF supernatant in phasphate buffered saline (PBS), pH 7.2 were incubated after addition of the antigen suspension for 18h at 37°C. The titer was the highest dilution showing a visible agglutination.

Partial purification of α-listeriolysin from bacterial culture supernatants:

Partial listeriolysin purification followed the method of Bhakdi et al. [36] . *L. monocytogenes* was cultured for 18 to 24 h at 37°C in 2 liters of Brain Heart infusion broth supplemented with 0.5% glucose. Cells were then removed by centrifugation and the supernatant was concentrated at 4°C . The listeriolysin was precipitated by the addition of 53 g solid ammonium sulfate / 100 ml with stirring for 30 min. at 4°C. The ammonium sulfate precipitate was collected by centrifugation, dissolved in approximately 10ml of PBS and dialyzed overnight against Veronal-buffered saline (pH 7.0 /4°C). Thereafter, listeriolysin was precipitated with 20% polyethylene glycol, the precipitate was dissolved in the Veronal- buffered saline and the material was applied to a diethylaminoethyl ether (DEAE) - sephacel column in the same buffer. The protein passing the column was pooled and directly utilized as the native toxin source Hemolytic titers of the partially purified toxin preparations were in the range of 2×10^3 - 4×10^3 U/ml.

Listeriolysin O assay :

LLO assay is based on the estimation of the hemolytic activity of the toxin activated with 20 mM cysteine towards human erythrocytes [37]. Measurement was made of the optical absorbance at 451 nm of haemoglobin released from erythrocytes (6×10^8 cells/ ml) incubated in PBS (pH 6.0) containing 0.1% bovine albumin (Sigma) . One hemolytic unit (HU) is the amount of toxin needed to release half the haemoglobin (50 % lysis) of the erythrocytes . It is estimated graphically by plotting lysis percent versus toxin volume on a long- probit graph.

The detection of specific anti-LLO in clinical samples :

Adsorption of Anti - SLO :

Because SLO and LLO are antigenically related [1], the detection of specific anti-LLO requires previous adsorption of Anti-SLO , which is often present at low titre in human body fluids [38] . Each human 0.5 ml sample (serum or CSF) , diluted 1/100 in PBS (pH 7.2) containing tween 20 (0.1%) and regilait (5%), was , then incubated for 1h at room temp. with SLO - adsorbed nitrocellulose filters.

Detection and titration of Anti- LLO :

The partial purified LLO was used as antigen for titration of anti-LLO- antibodies in clinical specimens (presumptive indirect haemagglutination reaction).

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Briefly , LLO was incubated for 15 min . at 37°C with increasing dilutions of body fluid sample (two-fold serial dilutions of samples were made in PBS containing 6mM cysteine , pH 5.8). 1/10 volume of a 10% solution of rabbit red blood cells was added and incubated for 45 min. at 37°C. The titre was the highest dilution inhibiting hemolysis (IU/ml).

RESULTS

Isolation and identification of *Listeria* :

Organisms which are Gram - positive and catalase - positive, which hydrolyze aesculin and exhibit " tumbling" motility at 20°C and 37°C, can be presumptively identified as *Listeria* spp. Characterization to species level is based on hemolytic activity on blood agar, the CAMP test (synergistic hemolysis with *S. aureus* but not with *Rhodococcus equi)* and the fermentation of carbohydrates (acid from glucose, D-salicin , L-rhamnose or α - methyl D-mannoside but no acid from D-xylose or D-mannitol). *L. monocytogenes* is also V. P. - positive but urease and oxidase non producers. API - Coryne System [30] was used to confirm the results of the conventional techniques for identification.

The obtained isolates of *L. monocytogenes* are shown in table (1) . The patterns of incidence of *L. monocytogenes* in patient groups (100%) was the same in both sera and CSF , meanwhile only 15% of the controls (30% of the non- healthy controls who had various foci of infection other than listeria) had also listerial infection.

Twenty six isolates of *L. monocytogenes* were obtained from both patient and control groups. These strains were subjected to be serotyped by a previously reported method [35] . Based on the agglutination reactions of listeria with highly absorbed rabbit antisera , a number of O and H antigens were identified and

Table (1): Incidence of *Listeria monocytogenes* in the studied cases.

Group / subgroup	Isolated *L. monocytogenes* from					
	Sera			CSF		
	Total	+	%	Total	+	%
Patients - pregnant	9.0	9.0	100	9.0	9.0	100
- Post abortive	14	14	100	14	14	100
Controls - married healthy	10	0.0	0.0	4.5	0.0	0.0
- Married non healthy	10	3.0	30	6.0	0.0	0.0
Total	43	26	60.5	33	23	70

Table (2) : Relative occurrence of serovars of *L. monocytogenes* in the 26 isolates.

Serovar	Number	%
1/2 a	5.0	20
1/2 b	2.0	8.0
1/2 c	1.0	2.0
4b	18	70

subdivided the isolated *L. monocytogenes* into many serovars. Table (2) shows only : 4 serotypes : 1/2 a , 1/2 b, 1/2 c and 4b could be obtained. Only two serotypes: 1/2 a and 4b are isolated in 90% of cases of human listeriosis.

Listeria O and H antibodies were detected in about 56% of pregnant women - sera with listeriosis while 100% and 78% respectively in CSF. The case is worse in cases of post abortive women with listeriosis where O antibodies were detected in 100% of both sera and CSF while 86% in serum and 93% in CSF for H antibodies. O and H - listeria antibodies were detected in only 30 and 20% , respectively of sera of the healthy married women control subgroup, meanwhile none could be detected in CSF . The other control subgroup of non- healthy married women recorded 50% and 20% of sera containing O and H antibodies, respectively, whereas only 33% of CSF containing O antibodies (Tables 3 and 4) .

By contrast , anti- LLO was detected in 100% of sera and CSF of patient group with titres ranged from 100 to > 800 . In non - healthy control subgroup , only 30% of sera contained anti- LLO with titres not more than 200 (Table 5) . Anti- LLO could not be detected in healthy married women control subgroup and also in CSF of the non healthy control subgroup .

DISCUSSION

Two major types of human listeriosis are recorded [5], materno faetal and adult - juvenile . In materno faetal listeriosis , a pregnant woman (maternal bacteraemia) develops a characteristic self-limiting "Flu - like" illness which may then lead , after a variable period of time to abortion, delivery of a stillborn child or the birth - often premature- of a child with neonatal listeriosis . In adults , listeriosis may present as a meningitis and sometimes as a septicaemic illness. Pregnancy, while predisposing to listeriosis , does not seem to predispose to carriage of the organism [39]. Maternal listeriosis can be associated with abortion late in the third trimester of pregnancy, but more commonly, infection presents as preterm labor[3]. Healthy pregnant

Table (3) : Listeria agglutinating antibodies in sera of patients and controls.

Group	Number with listeria anti- O/anti- H titre in sera							
	<40	40	80	160	≥320	Total	≥40	%
Patients with listeriosis :								
Pregnant	4/4	0/3	2/2	0/0	3/0	9	5/5	56/56
Post abortive	0/2	3/6	1/2	2/4	8/0	14	14/12	100/86
Controls married women :								
Healthy	7/8	2/1	1/1	0/0	0/0	10	3/2	30/20
Non healthy	5/8	2/1	2/1	1/0	0/0	10	5/2	50/20

Table (4) : Listeria agglutinating in CSF of patients and tested controls.

Group	Number with listeria anti- O/anti- H titre in CSF							
	<40	40	80	160	≥320	Total	≥40	%
Patients with listeriosis :								
Pregnant	0/2	1/3	3/2	2/0	3/2	9	9/7	100/78
Post abortive	0/1	0/3	2/0	2/5	10/5	14	14/13	100/93
Controls married women :								
Healthy	4/4	0/0	0/0	0/0	0/0	4	0/0	0/0
Non healthy	4/6	2/0	0/0	0/0	0/0	6	2/0	33/0

Table (5) : Anti- listeriolysin -O (Anti-LLO) in patience and controls clinical samples

Group	Number with listeria anti- O/anti- H titre in CSF													
	Serum							CSF						
	No.	<100	100	200	400	≥800	+%	No.	<100	100	200	400	≥800	+%
Patients with listeriosis :														
Pregnant	9	0	0	1	3	5	100	9	0	0	0	1	8	100
Post abortive	14	0	0	0	1	13	100	14	0	0	0	0	14	100
Controls married women :														
Healthy	10	10	0	0	0	0	0	4	4	0	0	0	0	0
Non healthy	10	7	2	1	0	0	30	6	6	0	0	0	0	0

women may be carriers of *L. monocytogenes* and still give birth to healthy infants. The relative risk of abortion of stillbirth due to *L. monocytogenes* is unknown and there is no concerete evidence that listeriosis is associated with repeated abortions or infertility [40].

Listeriosis is most frequently documented during the third trimester of pregnancy , however, cases have been confirmed as early as the second month of gestation [41]. As bacterial cultures are not routinely performed on spontaneously aborted faetuses and stillborn neonates , it is unclear whether listeriosis is a significant cause of early loss of the fetus. French researchers were able to grow *L. monocytogenes* from placental and fetal cultures in 1.6 % of pregnancies that resulted in premature labor and spontaneous abortion [42].

Although human listeriosis may be caused by all 16 serovars of *L. monocytogenes* , this study shares others [43-47] that only three serovars 1/2 a, 1/2b, and 2b cause most of the cases (98% in this study) .

Also, **Bruce et al.** [48] reported that despite the diversity of the 16 serotypes of *L. monocytogenes* , only three serovars are responsible for more than 90% of human disease. Gellin et al. [49] serotyped 161 *L. monocytogenes* isolates as 33, 31, 30 , 4, 1 and 0.5% to be 4b, 1/2b, 1/2a , 3b, 3a and 1/2c, respectively. Moreover, **Lacey**[5] reported that only three serotypes are isolated in more than 95% of cases of human listeriosis and these serotypes are also the most commonly isolated from food sources.

The immune response, cell biology of intracellular growth and bacterial determinants of the pathogenicity of *L. monocytogenes* still are being under evaluation. One likely determinant of pathogenicity is the elaboration of a sulfhydryl-activated hemolysis [50]. The observations that all strains of *L. monocytogenes* isolated from natural infections produce a zone of hemolysis on blood agar medium and that these strains are virulent in the mouse model, whereas non- hemolytic strains isolated after multiple subcultures [51] or from the environment are avirulent, first suggested that a hemolysin (Listeriolysin O) might be a relevant virulence factor [15]. The *L. monocytogenes* hemolysin (LLO) is a member of a family of bacterial pore- forming cytolysins of which streptolysin O is the prototype [52]. Listeriolysin O appears to be the first key factor for intracellular growth identified at the molecular level in *L. monocytogenes* [10].

The diagnosis of listeriosis requires the isolation of the organism but serological tests have not been shown to be useful tools , since *L. monocytogenes* has several antigens that cross - react with other Gram-positive organisms [53] and false- positives may occur [35,54]. Detection of anti- listeriolysin O in human body fluids may prove useful both for immunodignosis of the disease and for epidemiological studies [38]. Various products have been considered as virulence determinants in *L. monocytogenes* such as hemolysis (Listeriolysin -O), catalase, superoxide dimutase and the surface components referred to as monocytosis producing activity (MPA), immunosuppressive activity (ISA), the delayed type hypersensitivity protein (DTH) and protein P60. Of these , protein P60 [8] and listeriolysin O [10] are now recognized as essential virulence factors **Parrisius** and his associates [20] found that polyclonal antibodies raised against the isolated protein (listeriolysin -O) reacted not only with the homologous antigen but also with SLO and vice versa, antiserum of SLO also reacted with α- listeriolysin . The collective results conform to the general consensus that SLO and LLO are closely related [52].

Therefore, in this study , previous get ride of anti-SLO was done before titration of anti-LLO in body fluids. The extracellular hemolysin (LLO) produced by cultivation of a clinical *L. monocytogenes* isolate is purified and used as an antigen in the haemagglutination kit. Listeriolysin O (LLO) differed remarkably from the other sulfhydryl- activated toxins in that its cytolytic activity towards erythrocytes from various animals was maximum at low pH (- 5.5) and was undetectable at pH 7.0.

The present study attested the previous studies in that antibodies to LLO are produced during listeriosis and that detection of anti-LLO could be used as a diagnostic tool of this disease. Anti-LLO titres over 200 IU/ ml were recorded (100%) in the sera and CSF of 23 women patients with frequent abortion due to listeriosis. This is an indicator for the sensitivity and specificity of the indirect haemagglutination reaction of LLO test. Several methods in which killed bacterial suspensions were used as crude antigens have been proposed to detect specific listeria antibodies, including agglutination [54-58] and complement fixation tests [59], immunoprecipitation [60] and passive immunohemolysis [61] . Unfortunately, these methods are non- specific because of antigenic cross-reactivity between *L. monocytogenes* and other Gram positive bacteria, such as *Streptococci, Staphylococi, Enterococci* and *Bacillus spp.* [62] .

Furthermore, such methods lack sensitivity, they can not be used to effectively diagnose human listeriosis [56,58]. The lack of sensitivity of the agglutination test was confirmed in this study where only 55% of pregnant patient sera exhibited anti-O and H titres over 40 and only 86 % of the post abortive patients exhibited anti-H titres over 40 . On the other hand, more better results were obtained by using CSF samples .

The presence of Anti-LLO in non- healthy control subgroup (30% had titres ≤ 200) might be attributed to previous subclinical listeria infections and the frequent carriage of *L. monocytogenes* in stools of healthy people supports this view [63] . The finding that most or all patients produced specific anti-LLO after infection with *L. monocytogenes* has several implications. First, that enough LLO is produced *in vivo* during the infectious process to induce a detectable immune response. Second, the unexpected early appearance of Anti-LLO suggests that clinical expression of listeriosis in man appears after an incubation period , during which the immune system is triggered in such a high risk group of patients (frequent abortion). Finally, the production of antibodies against a major virulence factor (LLO) that is produced in vivo during the process of intracellular multiplication seems to be a good marker of clinical infection.

Human listeriosis was considered of relatively minor concern but recently , the incidence of individual cases of listeriosis increased in several countries and this , together with a series of food - borne outbreaks, caused *L. monocytogenes* to become of significant

public health importance [43,64] particularly in pregnant women and immunocompromized individuals [54,65].

Thus, detection of Anti-LLO might be reliable and useful for epidemiological surveys and for diagnosis of listeriosis, especially when bacteria have not been isolated. This is very important listeriosis marker in such high risky populations. The most common agents of congenital infections are toxoplasma, rubella, cytomegalovirus and Herpes simplex virus, the so- called TORCH agents [66]. This type of infections may also be caused by other microbes, as highlightened by this study, such as *L. monocytogenes*..

In conclusion, the study recommends the use of Anti-LLO marker as one of the pregnancy threatening diseases panel (TORCHL).

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التشخيص المناعى لمرضى اللستريا الحوامل

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إن زيادة معدلات الإصاب ببكتريا اللستريا فى الإنسان والحيوان مؤخراً جعلت هذا المرض مشكلة صحية جديرة بالاهتمام لانه واحد من أهم أسباب الأجهاض للحوامل . وقد أجرى هذا البحث لمحاولة ايجاد طريقة للكشف عن هذا المرض . وعليه فقد تم فصل ٢٦ عزلة للبكتريا اللستريا (٢٣ من ٢٣ مريضة تعانى من أجهاض متكرر + ٣ من بين مجموعة ٢٠ أصحاء) . وقد كشف البحث أن العزلات المذكورة ينتمى معظمها (٩٠٪) إلى فصيلتين فقط هما (٢/ ١ أ) ، (٤ ب) من فصائل بكتريا اللستريا مونوسيتوجنيز . وفى محاولة للكشف عن الأجسام المضادة من نوعى (O) , (H) لبكتريا اللستريا فى كلا من الدم والسائل النخاعى لحوامل مرضى بالالتهاب السحائى الليستري ، فقد دلت الفحوص أن ٥٦٪ من هؤلاء المرضى تحوى دماؤهم على الأجسام المضادة (O) , (H) بينما ١٠٠٪ ، ٧٨٪ منهن يحتوى السائل النخاعى لهن على الأجسام المضادة (O) , (H) على التوالى لبكتريا اللستريا مونوسيتوجينز . أما عن مجموعة السيدات اللاتى يعانين من الأجهاض المتكرر نتيجة الإصابة بهذا المرض فقد احتوت دماؤهن والسائل النخاعى لهن على الأجسام المضادة (O) , (H) بنسب ١٠٠٪ و ٩٠٪ على التوالى . ولايجاد طريقة أخرى قد تكون أكثر دقة فى تشخيص وتحديد شدة المرض فقد تمت محاولة الكشف فى الدم والسائل النخاعى عن الأجسام المضادة لإنزيم اللستريولزين أو (Anti - LLO) الذى يفرز بواسطة بكتريا اللستريا داخل أجسام المرضى وذلك باستخدام طريقة التجلط الدموى الغير مباشرة (IHR) وبناء على ذلك أجرى الأختبار على دماء والسائل النخاعى لـ ٢٣ من المرضى اللاتى يعانين من الإجهاد المتكرر وكذلك دماء ٢٠ والسائل النخاعى لـ ١٠ من الأصحاء . وقد أوضح البحث أن ١٠٠٪ من مرضى اللستريا وكذلك ١٥٪ من المجموعة الضابطة ممن يعانين من أمراض أخرى ليس من ضمنها اللستريا كانت نتائج الكاشف أيجابية للاجسام المضادة (Anti - LLO) بمعدلات (Titres) تراوحت بين أقل من ١٠٠ إلى أكثر من ٨٠٠.