Detection of Bacterial Endotoxin in Rabies Vaccine by the Gel-clot Assay with Tachypleus Amoebocyte Lysate

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ABSTRACT

The gel-clot assay with tachypleus amoebocyte lysate to detect the bacterial endotoxin in Rabies Vaccine (KMB17 cell) for Human Use (freeze-dried) is investigated in this paper. The test for interfering factors is performed to study the applicability of the gel-clot assay for detection of bacterial endotoxin in Rabies Vaccine (KMB17 cell) for Human Use (freeze-dried).The results suggest that when concentration of test solution is for Rabies Vaccine (KMB17 cell) for Human Use (freeze-dried), there is no interference on the congregation reactivity between tachypleus amebocyte lysate and bacterial endotoxin. The gel-clot assay with tachypleus amebocyte lysate can be used to detect the bacterial endotoxin in Rabies Vaccine (KMB17 cell) for Human Use (freeze-dried) due to its high sensitivity, its reliability and its simple handling.

Abbreviations: TAL, Tachypleus Amoebocyte Lysate

Introduction

Bacterial endotoxin is a high molecular weight complex (~10^6Da) of lipopolysaccharides (LPS) which constitute the major element of the outer cell wall of Gram-negative bacteria [1]. Endotoxin is a significant intended for parenteral in manufacturing processes by contaminant in biological products bioprocesses. Bacterial endotoxin has an extensive biological activities, which can generally induce a series of clinical responses when exposed on humans, such as fever, diarrhea,

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vomiting, septic shock, pyrogenicity, and disseminated intravascular coagul--ation[2]. Hence, endotoxin should be completely removed in the final product because of the high toxicity; the determination for amounts of remaining endotoxin is of great importance. The Limulus Amebocyte Lysate (LAL) assay is the gold standard as of today to detect endotoxin [3], and is widely used as a convenient method due to its high sensitivity, its reliability and its simple handling. There are three commonly available methods for endotoxin detection, i.e., gel-clot assay, kinetic turbidimetric assay, and chromogenic assay. In this paper, gel-clot assay is used to detect the amount of bacterial endotoxin in Rabies Vaccine (KMB17 cell) for human use (freeze-dried) at the developmental stage [4].

Materials and Methods

1. Materials: Two Tachypleus Amebocyte Lysate(TAL) reagents which labelled sensitivity (λ) is 0.25EU/ml were purchased. One(specification, 0.1ml; lot,0911101) is from Zhanjiang A＆C Biological LTD.; The other (specification, 0.1ml; lot,10081912) is from FuZhou XinBei Biological Industrial Co.,Ltd. The working standard endotoxin(WSE) and the water for bacterial endotoxin test (BET) are all from Zhanjiang A＆C Biological Ltd.

For the WSE, the specification is 10EU/vial and the lot number is 0901220. For the water for BET, the specification is 5ml/vial, the lot number is 0903050.All glassware are eliminated extraneous endotoxin by heating in a hot-air oven at 250℃ for 45min.The pipette tips for automatic pipetters are free of detectable endotoxin. The Rabies Vaccine (KMB17 cell) for human use (freeze-dried) is developed and manufactured by ZheJiang Pukang Biotechnology Co., Ltd. The specification of vaccine is 1.0ml, and the lot number is 20100401, 20100501 and 20100601.

2. Establishment of endotoxin limit: The endotoxin limit(L) of the Rabies Vaccine (KMB17 cell) for human use (freeze-dried) is 50EU/ml to ensure its safety for recipients in accordance with the Rabies Vaccine (Vero cell) for human use (freeze-dried) [5] .

3. Determination of the concentration at minimum valid dilution (MVD): The formula of minimum valid dilution(MVD) is

$MVD = cL/\lambda$.

For the Rabies Vaccine (KMB17 cell) for human use (freeze-dried), MVD is because it is sterilized powders for injection. Then the minimum valid dilution concentration will be calculated according to the formula,

$c = \lambda/L$.

(L is the endotoxin limit of the sample being examined, λ is the labeled sensitivity of TAL reagent (EU/ml) and c is the concentration of the test solution).

The highest sensitivity of LAL is 0.03 EU/ml in common. For the Rabies Vaccine (KMB17 cell), the concentration at minimum valid dilution is $0.6\times10^{-3} ml/ml$ according to the formula of $c = \lambda/L$, when the endotoxin limit (L) is 50EU/ml.

4. Confirmation for labeled sensitivi--ty of TAL reagent: The working stan--dard endotoxin (WSE) (10EU/vial) is dissolved by 1.0ml water for BET, mixed for 15min with a vortex mixer, diluted to make appropriate four serial dilutions at the concentrations of 2 λ (0.5EU/ml),1.0 λ (0.25EU/ml), $1/2$ λ (0.125EU/ml), $1/4$ λ (0.0625EU/ml). Each ampoules of LAL which sensitivity should be confirmed is dissolved by 0.1ml water for BET, and four replicates are made by added 0.1ml standard endotoxin solution at every level, two ampoules as negative control (NC) are added 0.1ml water for BET simultaneously. Then all the reaction mixtures are incubated at 37℃ for 60min. The measured sensitivity of TAL (λ_c) is calculated by the following expression.

$\lambda_c = anti\log\left(\Sigma X/4\right)$.

Where X is the log endpoint concentration that is the last positive result at the concentrations of endotoxin in serial dilutions.

5.The preliminary test for interfering factors: The concentration at minimum valid dilution is $0.6\times10^{-3} ml/ml$ according to the formula of $c = \lambda/L$, when the endotoxin limit(L) is 50EU/ml .The Rabies Vaccine(KMB17 cell) are reconstituted with 1.0ml water for

BET and diluted to make four serial dilutions at the concentrations of $10\times10^{-3}\,ml/ml$, $5.0\times10^{-3}\,ml/ml$, $2.5\times10^{-3}\,ml/ml$, $1.25\times10^{-3}\,ml/ml$.

Test solutions A and B are prepared , test solution A is the product solution being examined with no standard endotoxin solution; Test solution B is the product solution being examined with standard endotoxin solution (0.50EU/ml). Each ampoules of LAL are dissolved with 0.1ml water for BET; the group of negative product control (NPC) is then added 0.1ml solution A; the group of positive product control (PPC) is then added 0.1ml solution B. Negative control (NC) and positive control (PC) are set. NC are 2 ampoules with 0.2ml water for BET, and PC are 2 ampoules with 0.1ml water for BET and 0.1ml standard endotoxin solution (0.5EU/ml). The reaction mixtures are incubated at 37℃ for 60 min.

6. The test for interfering factors: The concentration of test solution for Rabies Vaccine is $0.5\times10^{-3}\,ml/ml$ in the test for interfering factors. Two kinds of TAL reagent (0911101 and 10081912) and three batches of Rabies Vaccine (20100401, 20100501 and 20100601) are used to test. Solutions A, B and C are prepared. Solution A is the test solution for Rabies Vaccine at the concentration of $5.0\times10^{-3}\,ml/ml$. Solution B contains test solution for Rabies Vaccine at the concentration of $5.0\times10^{-3}\,ml/ml$ .and standard endotoxin solution which concentration is 2λ(0.5EU/ml),1.0 λ (0.25EU/ml), $\frac{1}{2}$ λ(0.125EU/ml) and $\frac{1}{4}$ λ (0.0625EU/ml) respectively. Solution C is a series of standard endotoxin solution which concentration is 2 λ (0.5EU/ml),1.0 λ (0.25EU/ml), $\frac{1}{2}$ λ(0.125EU/ml) and $\frac{1}{4}$ λ (0.0625EU/ml). The test procedures are same with the preliminary the test for interfering factors.The geometric mean endpoint concentration of solution B(Et) and C(Es) are determined by the following formula.

$$E_s = anti\log\left(\Sigma X_s / 4\right) \qquad E_t = anti\log\left(\Sigma X_t / 4\right)$$

Where X_s is the log endpoint concentration of the solution C, and X_t is the log endpoint concentration of the solution B.

7. Detection of bacterial endotoxin in Rabies Vaccine (KMB17 cell) for human use (freeze-dried): The test procedures are same with the preliminary test for interfering factors. Bacterial endotoxin in Rabies Vaccine (20100401, 20100501 and 20100601) is detected at the concentration of $5.0\times10^{-3} ml/ml$.

Results

1. The result of confirmation for labeled sensitivity of TAL reagent: The labeled sensitivity (λ is 0.25EU/ml) of the two TAL reagents are confirmed. The test is valid, because all the four ampoules of the highest concentration (2λ, 0.5EU/ml) is positive, and all the four ampoules of the lowest concentration ($\frac{1}{4}$λ,0.0625EU/ml) is negative. All the measured sensitivity (λ_c) is between 2λ (0.5EU/ml) and $\frac{1}{2}$λ (0.125EU/ml) (Table 1).So the sensitivity of the two TAL reagents are 0.25EU/ml, i.e. the labeled sensitivity.

2. The result of the preliminary test for interfering factors: The preliminary test for interfering factors is performed to determine the concentration of test solution which is no interference with the gel-clot assay for bacterial endotoxin. The results suggest that concentration at $10\times10^{-3} ml/ml$ is no interference (Table 2).

Table 1: The result of confirmation of labeled sensitivity of TAL

Lot of TAL reagent	Manufacturer	Labeled sensitivity λ /(EU/ml)	Standard endotoxin solution/(EU/ml) 0.5 (2λ)	0.25 (1.0λ)	0.125 (½λ)	0.0625 (¼λ)	NC	Measured sensitivity λ_c /(EU/ml)
0911101	Zhanjiang A& C Biological LTD	0.25	++++	++++	−−−−	−−−−	−	0.25
10081912	FuZhou XinBei Biological Industrial Co.,LTD	0.25	++++	++++	−−−−	−−−−	−	0.25

"＋" positive result , refer to a firm gel formed; "－"negative result , refer to a firm gel not formed

Table 2: The results of the preliminary test for interfering factors

Lot of TAL reagent	Sensitivity λ /(EU/ml)	Experimental group	Concentration of test solution with rabies vaccine (ml/ml) 10×10^{-3}	5.0×10^{-3}	2.5×10^{-3}	1.25×10^{-3}	PC	NC
0911101	0.25	NPC	− −	− −	− −	− −	+ +	− −
		PPC	+ +	+ +	+ +	+ +		

"＋" positive result , refer to a firm gel formed; "－"negative result , refer to a firm gel not formed

3. Result of the test for interfering factors: The test for interfering factors is performed to study the applicability of the gel-clot assay for detection of bacterial endotoxin in Rabies Vaccine (KMB17 cell) for Human Use (freeze-dried) at the concentration of $5.0\times10^{-3}\,ml/ml$ .All the solution A and NC are negative. E_s is 0.25 all at the range of 0.5λ~2.0λ, and all the E_t/E_s

Table 3: The results of the test for interfering factors with TAL reagent from Zhanjiang A&C Biological Ltd.

Solution	Lot number of LAL	Sensitivity λ /(EU/ml)	Batch number of rabies vaccine	Concentration of test solution with rabies vaccine (ml/ml)	Standard endotoxin solution/(EU/ml) 0.5 (2λ)	0.25 (1.0λ)	0.125 (1/2λ)	0.0625 (1/4λ)	0	E_s	E_t	E_t/E_s
A	0911101	0.25	20100401	5.0×10^{-3}	/	/	/	/	— —	/	/	/
	0911101	0.25	20100501	5.0×10^{-3}	/	/	/	/	— —	/	/	/
	0911101	0.25	20100601	5.0×10^{-3}	/	/	/	/	— —	/	/	/
B	0911101	0.25	20100401	5.0×10^{-3}	++ ++	++ ++	+— ——	—— ——	/	/	0.21	0.84
	0911101	0.25	20100501	5.0×10^{-3}	++ ++	++ ++	++ ++	—— ——	/	/	0.125	0.50.
	0911101	0.25	20100601	5.0×10^{-3}	++ ++	++ ++	++ ——	—— ——	/	/	0.18	0.72
C	0911101	0.25	/	/	++ ++	++ ++	—— ——	—— ——	/	0.25	/	/
NC	0911101	0.25	/	/	/	/	/	/	— —	/	/	/

"＋" positive result , refer to a firm gel formed; "－"negative result , refer to a firm gel not formed

Table 4 The results of the test for interfering factors with TAL reagent from FuZhou XinBei Biological Industrial Co., Ltd.

Solution	Lot number of LAL	sensitivity λ /(EU/ml)	Batch number of rabies vaccine	Concentration of test solution with rabies vaccine (ml/ml)	Standard endotoxin solution/(EU/ml) 0.5 (2λ)	0.25 (1.0λ)	0.125 (1/2λ)	0.0625 (1/4λ)	0	E_s	E_t	E_t/E_s
A	10081912	0.25	20100401	5.0×10^{-3}	/	/	/	/	— —	/	/	/
	10081912	0.25	20100501	5.0×10^{-3}	/	/	/	/	— —	/	/	/
	10081912	0.25	20100601	5.0×10^{-3}	/	/	/	/	— —	/	/	/
B	10081912	0.25	20100401	5.0×10^{-3}	++ ++	++ ++	++ ++	—— ——	/	/	0.125	0.50
	10081912	0.25	20100501	5.0×10^{-3}	++ ++	++ ++	++ +—	—— ——	/	/	0.149	0.60.
	10081912	0.25	20100601	5.0×10^{-3}	++ ++	++ ++	++ ++	—— ——	/	/	0.125	0.50
C	10081912	0.25	/	/	++ ++	++ ++	—— ——	—— ——	/	0.25	/	/
NC	10081912	0.25	/	/	/	/	/	/	— —	/	/	/

"＋" positive result , refer to a firm gel formed; "－"negative result , refer to a firm gel not formed

are not less than 0.5 and not more than 2.0(Table 3 and 4).The results suggest that the test is valid and the interference from the test solution, prepared by Rabies Vaccine (KMB17 cell) for Human Use (freeze-dried) at the concentration of $5.0\times10^{-3}\,ml/ml$ is eliminated. The gel-clot assay with TAL can be used to detect the bacterial endotoxin in Rabies Vaccine (KMB17 cell) for Human Use (freeze-dried) when the concentration of test solution is not more than $5.0\times10^{-3}\,ml/ml$.

4. The results of detection of bacterial endotoxin in Rabies Vaccine (KMB17 cell): The positive control (PC) and positive production control (PPC) are positive; the negative control (NC) is negative. Hence, the test is valid. For the three batches of Rabies Vaccine (KMB17 cell), the negative product control (NPC) is negative (Table 5). The results suggest that the gel-clot assay is applicable to detect bacterial endotoxin in Rabies Vaccine (KMB17 cell) at the concentration of $5.0\times10^{-3}\,ml/ml$, and the amount of bacterial endotoxin is less than 50EU/ml.

Discussions

Given that TAL is composed of a series of coagulation enzymes, pH and temperature have a crucial influence over its reactions. The chelating agent EDTA, was found to inhibit endotoxin-induced LAL reaction, hence the importance of divalent captions to the integrity of the reaction [6]. Thus, components of the test sample could contribute to interference,

Table 5: The results of detection of bacterial endotoxin in Rabies Vaccine (KMB17 cell)

Batch number of rabies vaccine	Concentration of test solution with rabies vaccine (ml/ml)	Lot number of LAL reagent	Sensitivity λ/(EU/ml)	Experimental group		PC	NC
				NPC	PPC		
20100401	5.0×10^{-3}	0911101	0.25	− −	+ +		
20100501	5.0×10^{-3}	0911101	0.25	− −	+ +	+	−
						+	−
20100601	5.0×10^{-3}	0911101	0.25	− −	+ +		

"＋" positive result , refer to a firm gel formed; "－"negative result , refer to a firm gel not formed

therefore affecting the final result. When established the gel-clot assay with LAL for the Rabies Vaccine (KMB17 cell) for human use (freeze-dried) at the developmental stage, the test for interfering factors should be carried out.

For the Rabies Vaccine (KMB17 cell), the concentration at minimum valid dilution is $0.6\times10^{-3}ml/ml$ when the endotoxin limit (L) is 50EU/ml. In the preliminary test for interfering factors, the results suggest that the interference is eliminated at the concentration of $10\times10^{-3}ml/ml$ (Table 2). In this paper we select the concentration of $5.0\times10^{-3}ml/ml$ as the concentration of test solution in the test for interfering factors. Two LAL reagents and three batches of Rabies Vaccine are used to test. E_s is 0.25 all at the range of 0.5 λ ~2.0 λ , and all the E_t/E_s are not less than 0.5 and not more than 2.0(Table 3 and 4).The results suggest interference from the test solution, prepared by Rabies Vaccine (KMB17 cell) for Human Use (freeze-dried) at the concentration of $5.0\times10^{-3}ml/ml$ is eliminated.

Bacterial endotoxin in Rabies Vaccine (KMB17 cell) is detected. The results suggest that the gel-clot assay is applicable, and the amount of bacterial endotoxin is less than 50EU/ml (Table 5).

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