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DETECTION LIMITS OF METHODS USED IN STERILITY TESTING OF INACTIVATED VETERINARY VACCINES

By

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ABSTRACT

A total number of 63 samples represent 7 different inactivated veterinary vaccines each type was represented by 3 different batches and experimentally contaminated with 5, 20 and 100 CFU *Salmonella* Typhimurium reference strain /bottle then examined for sterility. Two ml from each batch was tested in comparison with different volumes of the same bottle ranging from 2ml up to 25 ml related to final vaccine volume as referenced by OIE. Further dilution of vaccines was done in a ratio1:10, 1:15 and 1:20 which might be attributed to formalin residues. Statistical analysis indicates no significant difference for the effect of two volumes on the validity of final results by all formalized and non-formalized vaccines, while there is significant difference between direct inoculation of test vaccine and different dilutions. Finally, it could be concluded that, the test method is appropriate for testing sterility of most inactivated vaccine types but some vaccines need further dilution before being tested.

INTRODUCTION

Vaccination is one of the more effective ways to protect animals from specific diseases. Disease producing microorganisms can be classified into small to large microorganisms as viruses, bacteria, fungi, protozoa and parasites. An inactivated or inactivated vaccine is a vaccine consisting of virus particles, bacteria or other pathogens that have been grown in culture and then killed using different methods such as heating or adding chemical substances as formaldehyde **(Gray, 2010).**

Inactivated or dead vaccines have very little risk of a live contamination while live vaccines always run the risk of contamination with unwanted organisms **(Tizard, 1995).**

Adequate assurance of sterility and freedom from contamination can only be achieved by proper control of the primary materials used and their subsequent processing and storage. Tests on the product are necessary to check that this control has been achieved **(OIE 2016 and CFR, 2015)**.

Most biological products including vaccines are required to be tested for sterility at the final container. So, sterility testing is designed to demonstrate the presence or absence of extraneous viable contaminating microorganisms in biologics **(Lee, 1990).**

The sterility testing method described in **CFR (2015)** is based on observation of turbidity in liquid culture media due to growth of potential contamination **(Parveen, 2011)**.

Limit of detection (LoD) is the lowest amount of analytic portion in sample that can be detected with stated probability, although perhaps not quantified as an exact value **(WHO, 2004).**The performance of a test is defined by selectivity, trueness, sensitivity, specificity, concordance, repeatability and reproducibility according to the requirements of **Excel kontrol (en) (2008) , Excel kontrol version 2.1 (2008)**.

The aim of this study is to assess the validity of procedures used for testing bacteriological sterility of veterinary inactivated vaccines when testing different volumes of the same analyze.

MATERIAL AND METHODS

Sampling:

Sixty-three inactivated veterinary vaccines representing 7 different types were selected and each type was represented by 3 different batches. All kinds of examined vaccines were in liquid phase and with different volumes ranged from 10ml to 250 ml. Each bottle of vaccine was experimentally contaminated with one of three different levels of aerobic bacteria 5, 20 and 100 CFU / vaccine bottle.

All volumes as indicated in (Table 1) the analytical unit (Test portion) was examined for detection of bacterial contaminations in 2 ml and compared sample volume indicated by **OIE (2016),** briefly 2ml in vaccine bottle ranged from 4 - 20ml, and10 % of total volume of vaccine bottle ranged from >20ml up to 250ml.

The study was performed on each spiked bottle of vaccine by direct inoculation and three levels of dilution 1:10, 1:15 and 1:20 tested as well.

Table (1): Classification of inactivated vaccines used for determination of validity of sterility test applied.

Vaccine	Code no	Inactivator used	Adjuvant	Total volume	Test portion	
					Lab.	OIE
Inactivated bacterial poultryvaccine	1	Formalin	-	100ml	2ml	10ml
Inactivated viral poultry vaccine	4 5	Formalin Non-formalized	-	30ml 250ml	2ml 2ml	3ml 25ml
Inactivated bacterial large animal vaccine	2	Formalin	Alum OH gel	100ml	2ml	10ml
Inactivated viral large animal vaccine	3 7	Formalin Non-formalized	Alum OH gel	100ml 20ml	2ml 2ml	10ml 2ml
Inactivated combined (bacterial and viral) large animal vaccine	6	Formalin	Oil in gel	10ml	2ml	2ml

Culturing media:

Thioglycolate broth and Tryptic soya agar were used for detection of aerobic bacteria contamination **(OIE, 2016**).

Reference strains:

Salmonella Typhimurium reference strain was kindly obtained from Reference Strain Bank, Central Laboratory for Evaluation of Veterinary Biologics (CLEVB).

Preparation of the inoculums (Reynolds, 2005).

Enumeration of test organism in the inoculums:

Three beads of *Salmonella* Typhimurium were enriched in three tubes 10ml of buffer peptone water at 37°C overnight 16-18 hrs. to produce a culture containing approximately 9 Log_{10} CFU/ml. These broth cultures were serially tenfold diluted in Maximum Recovery Diluents (MRD) in order to find the theoretical number of *Salmonella* will be added to vaccine bottle. ml aliquot of appropriate dilution 7 Log_{10} of *Salmonella* culture was spread onto XLD, 10 times before usage, the mean was 12 CFU/0.1ml and used as theoretical values in the preparation of different inoculums.

Levels of contamination:

The overnight culture broth of the target organism was diluted serially so that inoculums contained the desired number (expressed by colony forming units "CFU" per vaccine bottle) of test organism as:

Low level 5 CFU/ vaccine bottle.

Medium level 20 CFU/vaccine bottle.

High level 100 CFU/vaccine bottle.

Method used:

All tested vaccines were in liquid phase and with different volumes ranged from 10ml to 250 ml and spiked with prepared aerobic strain culture of *Salmonella* Typhimurium with 3 different levels of contamination (5 CFU/bottle, 20 CFU /vaccine bottle and 100 CFU/vaccine bottle).

Test portion was performed in two ways, first way regardless to vaccine volume; 2 ml from each spiked vaccine was used, while the second way was according to **OIE (2016)**, 2ml from vaccines ranged from 4-20 ml and10 % of total volume of vaccine volumes more than 20 ml up to 250 ml.

All test portions under examination were added to thioglycolate broth and incubated at 35^{o}C for 14 days in ratio 1:15 in order to be examined for detection of *Salmonella* Typhimurium contamination.

Any suspected turbidity in medium containing the spike was examined by sub inoculation into tryptic soya agar medium for detection of the colonies.

All spiked vaccines were futherly diluted in a ratio 1:10, 1:15 and 1:20 then examined again for contamination.

Quality assurance:

Quality assurance is important for verification of the accuracy and precision in the formation obtained from analysis ensuring that, the data obtained from analysis are suitable for use in decision making, ensuring the correctness of data and ensuring proper functioning to decrease maintaining equipment failure.

Performance of equipment:

According to **ISO/7218 (2017**) all used equipment and monitoring devices were kept clean and in good working condition before use, calibrated to traceable national standards and according to working conditions and the accuracy demanded for the results.

Environmental monitoring (ISO/7218, 2017):

The temperature was checked periodically; the microbiological quality of air was checked before beginning of the study. The microbiological quality of surface was checked before analysis by using swab technique at acceptable limit <20 CFU/plate.

Media and reagent performance:

According to **ISO /11133-1 (2014)** media used in this study were tested before inoculation to validate their efficacy by measuring the productivity and selectivity.

Criteria of validation:

The validation study depends on different parameters and tools to evaluate if the method is fit for use in check vaccines sterility or not. The parameters used were selectivity, trueness, sensitivity, specificity, concordance, repeatability and reproducibility according to the requirement of ISO 16140. Additionally, the study included false negative results in vaccines number 1,2,3,7, and subjected for further dilution by percentage of 10 %, 15% and 20 % by taking 10 ml, 15 ml and 20 ml and completing to the original volume of the vaccine by using sterile saline, then the previously diluted vaccines were spiked with different levels of contaminations 5, 20 and 100 CFU and examined.

The parameters performed were selectivity, trueness, sensitivity, specificity, concordance and repeatability according to the requirement of **ISO/TS 34/SC 9/W 03/ 16140 (2017).**

Trueness = (a+d)/n *100

Sensitivity = a / (a+b) *100

Specificity = d/(c+d) *100

Concordance-Index Kappa=2 · (ad - bc) {(a +c) (c +d) + (a +b) (b +d)}.

Where:

a: Number of positive agreements.

b: Number of false negatives.

c: Number of false positives.

d: Number of negative agreements.

n: Total number of results.

Repeatability (r) =

Where:

x = Number of corresponding results under repeatability conditions

n = Number of tested samples.

Table (2): Criteria used for evaluation of test methods according to Concordance-Index Kappa: According to ISO/TC 34/SC 9/WG 3N 027.

Kappa Concordance	Assessment
<0.10 (No)	**Not Accepted [Not fit for use]**
0.10 – 0.40 (Low)	**Not Accepted [Not fit for use]**
0.41 – 0.60 (Distinct)	**Not Accepted [Not fit for use]**
0.61 - 0.80 (High)	**Accepted [Fit for use]**
0.81 - 1.00 (Almost Total)	**Accepted [Fit for use]**

RESULTS AND DISCUSSION

The samples under examination were categorized into 5 formalin inactivated (vaccines1, 2, 3, 4 and 6) with different volumes100,100,100,30 and 10 ml respectively, and 2 non-formalized (Vaccines 5 and 7) with volumes 250 and 20 ml respectively. Statistical analysis indicated no significant difference for the effect of two volumes on the validity of final results by all formalized and non-formalized vaccines, while there is a significant difference between direct inoculation of test vaccine and different dilutions applied.

As shown in (Table 3),vaccine No.6 [A large animal Formalized combined (bacterial and viral)] specificity, sensitivity and trueness are the same, at three levels of contaminants (5/20/100 CFU/vaccine bottle) and two tested portion volumes. The result indicates validation criteria according to [**ISO/TC 34/SC 9/WG 3N 027**] with 1 Kappa Index. So the method applied is appropriate for use for that kind of formalized vaccine.

Table (3): Criteria of validation study for Vaccine No.6 [combined (bacterial and viral) formalized large animal vaccine].

Validation Criteria					
Vaccine	**Specificity**	**Sensitivity**	**Trueness**	**Kappa Index**	**Assessment**
Vaccine No.6 [Direct Inoculation at three level of spike (5/20/100 CFU/vaccine bottle) and two test portion volume	**100**	**100**	**100**	**1**	**Good [Accepted]**

Table (4): Criteria of validation study for Formalized Vaccine No.1, 2, 3 and 4*

Vaccine	**Validation criteria**				
	Specificity	**Sensitivity**	**Trueness**	**Kappa Index**	**Assessment**
Vaccine No.1,2,3&4* [Direct Inoculation at three level of spike (5/20/100 CFU/vaccine bottle) and two test portion volume	**100**	**50**	**57.1429**	**0.2222**	**Low Not Accepted [Not fit for use]**

*** Vaccine No.4 at level of spikes 5 and 20 CFU/vaccine only.**

Table (5): Criteria of validation study for Formalized Vaccine No. 4 by using two kind of test portion at level of Spike 100 CFU/vaccine bottle direct inoculation.

	Validation criteria				
	Specificity	**Sensitivity**	**Trueness**	**Kappa Index**	**Assessment**
Vaccine No.4	**100**	**100**	**100**	**1**	**Accepted**

As shown in (Table 4) for vaccines No.1, 2, 3 and 4 (No.1 was inactivated bacterial poultry vaccine, No.2 was Inactivated bacterial large animal vaccine, No. 3 was Inactivated viral large animal vaccine and No. 4 was Inactivated viral poultry vaccine) indicates that, the validation criteria according to ISO/TS 34/SC 9/W 03 /16140 (2017) specificity, sensitivity, trueness and Kappa Index were 100, 50, 57.14 and 0.22 respectively for all results types of vaccines in case of direct inoculation.

At level of contamination at 100 CFU vaccine No.4 showed 100, 100,100 and 1 for specificity, sensitivity, trueness and Kappa Index respectively (Table 5 contamination) which reveals the appropriate of method used for this type of vaccine at high level of contamination.

So it can be concluded that, the test methods applied to check the sterility is appropriate for formalized vaccine No.6 (Inactivated combined bacterial and viral vaccine used for large animals). In spite of other formalized vaccines represented in vaccines number 1, 2, 3, 4 with volume 100,100,100, 30 respectively did not give any positive results and the criteria of validation (Kappa Index) was low [Not fit for use] which may be due to effect of the inactivator substance used at the three level of contamination.

The inactivator substance used in preparation of inactivated vaccine may be has inhibition effect on aerobic bacteria added. So, the study is designed to subject to make extra dilution of 10%, 15% and 20% then spiked.

As mentioned in (Tables 6 , 7) the results indicated that, the specificity, sensitivity, trueness and Kappa Index for vaccines number 2, 3 and 4 were 100, 100,100 and 1 respectively at two test portion volumes and three level of spike (contamination) while vaccine No.1 (inactivated bacterial poultry vaccine) gave negative results for all volumes. So, the in activators effect on contaminants for vaccines 2, 3 and 4 were negative and when diluted resulted in positive detection so the method fit for use for that kinds of vaccines in case of performance of decimal dilution at least 10 %. Vice versa for vaccine No.1 the specificity, sensitivity, trueness and Kappa Index were 100, 50, 57.1429 and 0.2222 as shown in (Table 7). So, the method is not appropriate for that kind of vaccine even after dilution which may be attributed to excess of formalin.

Table (6): Criteria of validation study for Vaccine No. 2,3and 4 and [Formalized vaccine] at 3 dilution factors 10%,15% and 20 % at three levels of Spike 5, 20 and 100 CFU/vaccine bottle direct inoculation.

	Validation criteria				
	Specificity	**Sensitivity**	**Trueness**	**Kappa Index**	**Assessment**
Vaccine No. 2, 3 and 4	**100**	**100**	**100**	**1**	**Good [accepted and fit for use]**

Table (7): Criteria of validation study for Vaccine No. 1 [Formalized vaccine] at 3 dilution factors 10%,15% and 20% at three levels of Spike 5 ,20 and 100 CFU /vaccine bottle direct inoculation.

	Validation criteria				
	Specificity	**Sensitivity**	**Trueness**	**Kappa Index**	**Assessment**
Vaccine No.1	**100**	**50**	**57.1429**	**0.2222**	**Low, Not Accepted [Not fit for use]**

The results obtained from (Tables 8, 9) for vaccine No.5 [Non Formalized Inactivated viral poultry vaccine] and vaccine No.7 [Inactivated viral large animal vaccine], validation criteria specificity, sensitivity, trueness and Kappa Index revealed that direct inoculation method is unable to detect the contamination while it could be detected easily after vaccine dilution. So, preparation of test sample is necessary before testing this type of vaccine.

Table (8): Criteria of validation study for Vaccine No.5[Non Formalized vaccine] Direct inoculation at Dilution factor 10 %,15% & 20% at three level of Spike 5 ,20 and 100 CFU/vaccine bottle direct inoculation.

	Validation criteria				
	Specificity	**Sensitivity**	**Trueness**	**Kappa Index**	**Assessment**
5 CFU	**100**	**50**	**57.143**	**0.22**	**Low**
20 CFU	**100**	**66.66**	**72.72**	**0.22**	**Low**
100 CFU	**100**	**75**	**80**	**0.55**	**Distinct**
Vaccine No.5 At Dilution factor 10,15 and 20 % at three level of spike	**100**	**100**	**100**	**1**	**Good**

Table (9): Criteria of validation study for Vaccine No.7 [Non Formalized vaccine] Direct inoculation at Dilution factor 10,15 & 20 at three level of Spike 5 ,20 and 100 CFU/vaccine bottle direct inoculation.

	Validation criteria				
	Specificity	**Sensitivity**	**Trueness**	**Kappa Index**	**Assessment**
Direct inoculation at three level of Spike	**100**	**50**	**57.14**	**0.22**	**Not accepted**
Vaccine No.7 At Dilution factor 10,15 and 20 % at three level of spike	**100**	**100**	**100**	**1**	**Good Accepted [fit for use]**

Finally, it could be concluded that, the test method is appropriate for testing sterility of most inactivated vaccine types but some vaccines need further dilution before being tested.

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حدود الكشف للاختبارات المستخدمة فى الكشف على نقاوة اللقاحات البيطرية المثبطة

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الملخص العربى

أجريت الدراسة على 63 زجاجة لقاح من سبعة أنواع مختلفة من اللقاحات البيطرية المثبطة والتى تمثل ثلاثة تشغيلات مختلفة لكل نوع .كل زجاجة لقاح تم تلويثها بثلاثة مستويات مختلفة (5, 20 and 100 CFU/bottle) من السالمونيلا تيفيميوريم ثم اختبارها باستخدام كميتين مختلفتين للاختبار إحداهما 2 مل والاخرى تتراوح بين 2 مل الى 25 مل حسب المكتب الدولى للأوبئة OIE تم تخفيف اللقاحات بنسب 10:1، 15:1، 20:1 لمقاومة تأثير الفورمالين على البكتريا المستخدمة في التلوث ثم إعادة اختبار النقاوة. التحليل الاحصائى للنتائج اشار لعدم وجود اختلاف معنوى بين كميتين الجزء المختبر من كل لقاح بينما كان هناك اختلاف معنوى بين استخدام اللقاحات مباشرة او بعد تخفيفها.