

## RESEARCH ARTICLE

### Comparative ELISA and SAT assays for the detection of *Ornithobacterium rhinotracheale* antibodies in broiler chickens at Sharkia Governorate

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#### Abstract

The *Ornithobacterium rhinotracheale* (ORT) has been involved in respiratory disease complex in poultry. In a trial to investigate the inclusion of ORT in broiler chickens suffering from respiratory troubles, two hundred and fifty birds (30 flocks) were subjected to clinical, postmortem (PM) and serological examination. The clinical findings of the examined birds were nasal discharges, rales, swollen eyes accompanied by diarrhea in some cases. Fibrinous pericarditis, perihepatitis and air sacculitis as well as caseated materials in trachea and bronchi were the frequently associated gross lesions beside foamy yoghurt like exudates. Enzyme linked immunosorbant assay (ELISA) was applied on sera samples (N= 250) to detect antibodies against ORT. ELISA results were positive among 80% of the tested flocks. A selected 49 sera samples were submitted for both ELISA and Slide agglutination test (SAT). Their outcomes revealed 93.9% positive for ELISA and 89.8% and 69.38% against ORT-II and ORT-I antigens respectively among chicken broilers (9-45) days of age. The comparable findings proved that in case of unavailability of ELISA assay, the SAT could be helpful alternative in ORT antibody monitoring.

**Keywords:** ORT, chickens, antibodies, ELISA, SAT.

#### Introduction

Chicken meat is the second greatest consumed meat and represents about 36% of world meat production. Therefore, poultry respiratory diseases are not only a problem for the chief producers, but also a global concern. Respiratory diseases in poultry are mostly accompanied by heavy economic losses and increased mortality rates, medication costs, and condemnation rates due to air-sacculitis and decreases in egg production. These infections have been recognized in many countries worldwide. Since 1980s up to the early of the 1990s, The *Ornithobacterium rhinotracheale* (ORT) was isolated from turkeys, Pekin ducks and chickens suffering from pneumonia, air sacculitis as well as other

respiratory diseases [1]. In 1991, it was observed that respiratory disease in broilers was associated with isolation of ORT, which was firstly detected ORT in Germany from Turkey flocks with respiratory problem [2,3].

The ORT has been incriminated in respiratory disease complex in birds [4]. Environmental; high-density confinement of birds causes behavioral problems and physical injury to birds. It is also related to stress in birds due to movement restriction and the unhealthy conditions generated by crowding, leading to a reduced air quality, which contributes to the onset of respiratory tract diseases, secondary infections may increase the severity of ORT [5]. ORT is a Gram-negative, non-mobile, non-sporulating

bacterium belonging to the superfamily V rRNA and family Flavobacteriaceae. It is from the Cytophaga-Flavobacterium-Bacteroides descending genetic line. ORT was previously designated as Gram-negative pleomorphic rod Pasteurella-like, Kingella-like, or Taxon 28 [1-3]

Enzyme linked immunosorbant assay (ELISA) has been successfully used for screening and detecting maternal antibodies in 1-day-old chickens and turkeys. Moreover, using ELISA antibodies against ORT can be detected in serum and egg yolk shortly after infection. Titers will peak between one to four weeks post infection [1]. Eighteen (A-R) serotypes of ORT were determined by ELISA with the majority of A,B,D and E. Field surveys for self-made ELISA or commercial ELISA kits were shown to be a useful tool for monitoring flocks [1,5,6]. The SAT versus various ORT antigens could also be used for serological examination [7]. In Egypt, the first detection of ORT in chicken broilers was carried by El-Gohary and Awaad [8]. At the same time, Youssef and Ahmed [9] detected the antibodies against ORT in 30–38-week-old breeders. Since that date increased interest was gained by Egyptian authors among ORT [8, 9-14]. Therefore, the objective of present work is to investigate the occurrence of antibodies against ORT in broiler flocks through using ELISA and SAT.

## Materials and methods

### *Clinical and postmortem examination*

A total of 250 birds representing 30 broiler flocks and backyard rearing (3-16 birds/ each) of different ages (9-45 days of age) suffering from respiratory problems were examined. Mortality rate was calculated at collection day; all flocks were vaccinated [Infectious bronchitis (IB primer) at day one, Newcastle diseases (ND + (Hitchner, HB1) at day five, then infectious bursal disease (IBD) day 15 while IB (Mas) +Clone 30 at day 18 of age] except backyard were not vaccinated. The

collected birds were examined for clinical signs and PM lesions.

### *Blood collection for serum preparation*

Two hundred and fifty blood samples were collected from the examined birds for serological investigation. Blood samples were collected without anti-coagulant, the sera were separated and collected in sterile Eppendorf tubes then stored at -20°C till using.

### *ELISA assay*

Reagents and chemicals used for ELISA test were prepared according to manufacturer company (Biochek; product code: CK 108, Lot No: FS6076). ORT plates were coated with inactivated antigen. Each sample was diluted 1:100 (1ml serum: 99M diluents) while Positive [antibodies specific to ORT in phosphate buffer with protein stabilizers and sodium azide preservative (0.1% W/V)] and negative [specific pathogen free serum in phosphate buffer with protein stabilizers and sodium azide preservative (0.1% W/V)] controls did not be diluted. Conjugate reagents consisted of anti-chicken alkaline phosphatase in Tris buffer with protein stabilizers, inert red dye and sodium azide preservative (0.1% W/V) were supplied. The contents of one wash buffer sachet were dissolved into one liter of distilled water. Then were justified to temperature at 22-27°C. Substrate tablets consisting of PNPP (P-Nitrophenyl phosphate) tablets were dissolved with substrate buffer followed by using of Stop solution (sodium hydroxide in diethanolamine buffer). All reagents were freshly prepared/day of use. The microtiter plate ELISA reader was used for reading of the absorbance of controls and the samples.

### *Test procedure*

- 1-ORT coated plate was removed from sealed bag to be labeled before performing the test.
- 2-The 100 µl of negative control serum was added into wells A1 and B1.

- 3-The same amount (100 µl) of positive control serum was added into two next wells C1 and D1.
- 4-One hundred µl of diluted samples were added into all wells and cover Plates with incubation at room temperature for 60 minutes.
- 5-Good aspiration to all contents of wells and washed with wash buffer) 350 µl per well 4 times following by good drying by inverting plates firmly on absorbent paper.
- 6-The conjugate reagent (100µl) was added into wells and covered then incubated at 22-27°C for 60 minutes.
- 7- Good washing for plates was repeated as in step 5.
- 8-The 100 µl of substrate reagent was added into the appropriate wells and covered plates with lids and incubated at room temperature for 30 minutes.
- 9-The 100 µl of stop solution was added to appropriate wells to stop reaction.
- 10- The micro titer plate reader was blanked on air and recorded the absorbance of controls and the samples by reading at optical density (O.D) 405 nm.

By reading at O.D 405 nm and Calculation of antibody titer was applied (according to manual instructions (Log 10 titer = 1.75(log 10S/P) +3.156).

#### **Mean of test sample-mean of negative control**

Mean of positive sample-mean of negative control.

Samples with an s/p of **1.0** or greater considered positive or contain antibodies for ORT.

For the good result of the mean negative control absorbance should below 0.30.

The greater than 0.15 was considered in the difference between the mean negative control and the mean positive control.

The test was carried out at room temperature.

#### **Quality control results**

Mean negative control Optical density = 0.157

Mean positive control Optical density = 0.837

#### **SAT assay**

Preparation of *brain heart infusion (BHI) agar (Oxoid-CM1136-UK corporate)*

It was used for the isolation of ORT, containing gentamycin at concentration of (10µg/ ml) according to Hafez *et al.* [3] to inhibit growth of other bacteria as *E. coli*, *Proteus sp.* and *pseudomonase sp.* Two references' ORT I and II were obtained from Ismail [15]. An amount of 37gm of brain heart powder and 6gm agar were added to 1 liter of distilled water on flask then and autoclaved at 121°C for 30 minutes and before pouring gentamycin was added.

The two reference of ORT strains were cultured on BHI agar separately at 37°C, 48h with 10% CO<sub>2</sub> for each plate.

#### **Preparation of inactivated antigen of ORT**

The cultivated plates were flooded using PBS and the colonies were collected in washing flask then the matrix agitated in sterilized tubes then centrifuged for 50 minutes, the final pellets were re-suspended in PBS/ 10<sup>9</sup> McFarland standard tube then inactivated with 0.5% formalin according to [16]

#### **SAT application and interpretation**

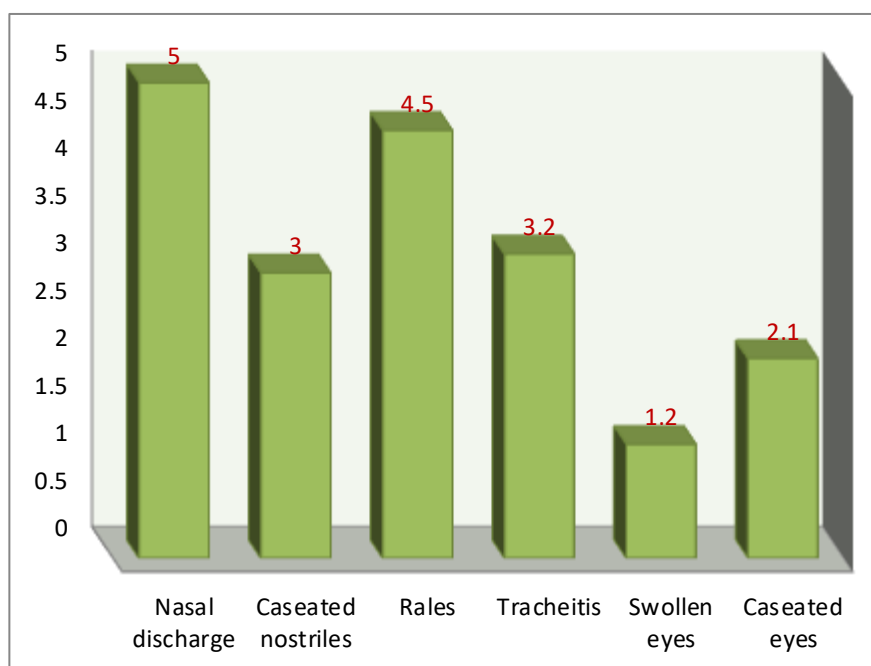
The amount of 25µl of inactivated antigen was added to tested samples (25µlof known serum) on a clean and sterilized slide then mixed. The reaction of agglutination was carried out and reading results within 1-2 minutes and must be recorded as a number of (+) according to the intensity of agglutination [7].

#### **Results and discussion**

Respiratory complications are the foremost illnesses facing chicken broilers leading to massive economic losses particularly in concurrency of other animate and inanimate agents [17-20]. The ORT is one of bacterial agents involved in respiratory diseases in chickens, which can cause highly affected birds contagious disease among affected birds. Consequently; the main objective of this study was to estimate the seropositive broiler chickens against ORT in Sharkia Governorate. For this purpose, 250 birds were clinically and serologically investigated.

The inspected birds were suffering from respiratory troubles "nasal discharge (100%), rales (83%), swollen eyes (23%). The declared respiratory troubles are nonspecific but were recorded among ORT infected birds [14, 17, 22-24] accompanied by diarrhea 46% of the examined flocks which could be attributed to concurrent

bacterial and / or viral infections [3, 25]. Pathological examination of the euthanized chickens revealed gross lesions including; caseated materials in eyes (33%), Fibrinous pericarditis, perihepatitis and air sacculitis (30%) as well as caseated materials in trachea and bronchi (63%), foamy yoghurt like exudates was recorded in 10%. Similarly Hafez *et al.* [15]; Sakai *et al.* [26] and El-Gohary *et al.* [21] reported foamy yoghurt like exudates in abdominal air sacs as a characteristic lesion among ORT infected chickens. On the other had, septicemia was observed (43%). These lesions could be explained by involvement of ORT in complicated respiratory disease (CCRD) of chicken's broilers as reported by many authors [25, 27]. Regarding the respiratory findings there was a good relatedness with the observed pm lesions (Figure 1). In the same consequences such relation was evidenced in previous studies [14, 20].



**Figure 1: Relatedness of clinical signs and gross lesions in examined chicken broilers.**

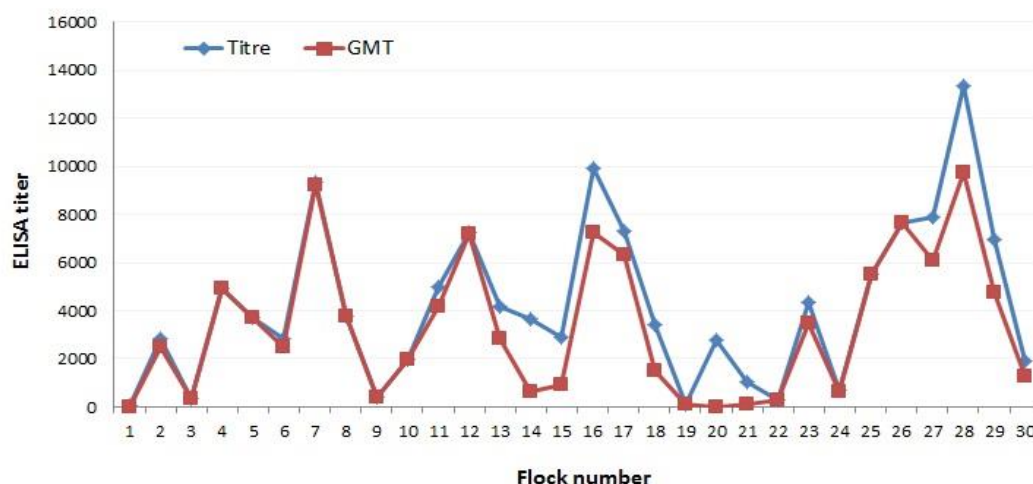
This supported the results in this study relevant to variable disease patterns and mortality rates (1-50%). Being, one of the biggest Governorates producing chickens in Egypt, Sharkia Governorate is located at the eastern Nile delta, and poultry farms are

stocked in a closely neighboring manner with inter lactated backyard rearing of mixed species. Flocks are exposed to frequent multiple infections including ORT.

Meanwhile the clinical signs and necropsy findings of ORT are not adequately specific for diagnosis, laboratory methods are needed for certain diagnosis [28]. For this purpose ELISA and SAT tests were carried out on collected sera of the formerly examined birds.

The results of ELISA revealed positive sera conversion in 24 flocks (80%); ELISA titer 1432 or greater/each sample was

considered positive for ORT antibodies. The range of mean antibodies titers was 1091-13343 and GMT 11-9749 (Fig. 2). Similarly Canal *et al.* [29] investigated the prevalence of antibodies against ORT in broilers in Southern Brazil and they found 63.83% positive sera in broilers at slaughter time. They also reported that there was a positive correlation between the presence of respiratory signs and antibodies to ORT.



**Figure 2: The ORT antibodies in Cobb chicken broilers using ELISA test.**

Also, El-Gohary and Sultan [11] detected 70.5% positive sera in broilers against ORT using ELISA while Refai *et al.* [13] detected ORT antibodies in 20.3-77.7% serum samples of broiler. Sixty three Sixty three percentage of the examined sera of broilers in Thailand were positive to antibodies against ORT while in Azarbijan 82% were positive [29].

In the present study, it was considered positive result at ELISA titers 1432 or greater as recommended by the manufacture proceeding. It was remarkable that there are titers as high as 13343 which may be explained by increased ORT bacterial load in the surrounding environment, since all examined flocks experienced no vaccination against ORT. Comparable titers  $10^{9.29}$  were recorded in Southern Brazil and they considered that flocks are positive when there was a minimum one positive bird [6].

Two reference antigens ORT-I and ORT-II [16] were used in SAT test to detect the antibodies in sera of suspected birds in this study. The results revealed positive seroconversion against ORT-I and ORT-II were positive in 34 (69.38%) and 44 (89.80%) serum samples respectively with in comparison to 46 positive serum samplers for ELISA (93.9%) among the same selected 49 serum samples (Figure 3 and Table 1). The economic impact of the disease is correlated to the severity of the clinical findings, duration of the disease and mortality which have found to be variable from being primary or secondary infection, adverse environmental factors and immune status of the birds may induce a range of disease [27].

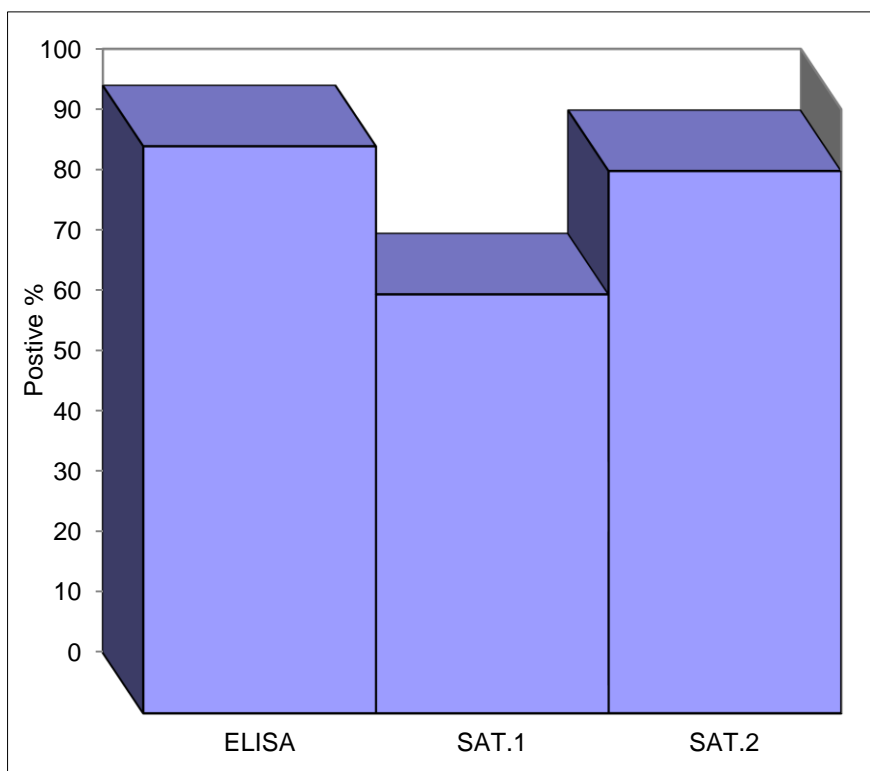
**Table (1): Comparative ORT antibodies detection using ELISA and SAT tests in selected chicken flocks.**

Flock No	Sample No	ELISA Titer*	SAT		Flock No	Sample No	ELISA Titer*	SAT	
			ORT-I	ORT-II				ORT-I	ORT-II
1	1	-	-	-	13	1	+	+	++
	3	-	-	-		2	+	Nd	Nd
2	3	+	+++	+++	14	3	+	-	+
	4	-	Nd	Nd		1	+	-	+
3	3	-	Nd	Nd	15	5	+	++	+++
	1	-	Nd	Nd		4	+	+	++
	2	-	Nd	Nd		6	+	+	++
4	1	+	Nd	Nd	5	+	Nd	Nd	
5	1	+	+	++	8	+	Nd	Nd	
6	2	+	+	++	9	-	Nd	Nd	
7	1	-	Nd	Nd	14	-	Nd	Nd	
	2	+	+	++	15	+	Nd	Nd	
8	1	+	+	++	16	2	+	Nd	Nd
	2	+	-	+		5	+	-	+
9	1	-	Nd	Nd	17	2	+	++	+++
	2	+	-	+		4	+	-	+
10	1	+	Nd	Nd	1	+	-	+	
11	1	+	+	++	6	+	+	++	
	2	+	-	+	7	+	++	+++	
	3	+	-	+	8	+	+	+++	
12	1	+	-	+	11	11	+	++	++
	2	+	-	+					

**Table 1 to be continued;**

Flock No	Sample No	ELISA Titer*	SAT		Flock No	Sample No	ELISA Titer*	SAT		
18	7	+	++	+++	21	4	-	Nd	Nd	
	5	+	++	+++		6	-	Nd	Nd	
	3	+	++	+++		22	1	-	Nd	Nd
	8	+	++	+++			2	-	Nd	Nd
	6	+	++	+++			3	-	Nd	Nd
19	2	-	-	-	23	4	-	Nd	Nd	
	1	-	Nd	Nd		1	+	+	+	
20	10	-	Nd	Nd		2	+	+	+	
	18	-	Nd	Nd		3	+	+++	+++	
21	1	+	-	+		4	+	+	++	
	5	+	-	+	5	+	+	++		
	1	-	Nd	Nd	24	7	-	Nd	Nd	
3	-	Nd	Nd	6		-	-	Nd	Nd	
Flock No	Sample No	ELISA Titer*	SAT		Flock No	Sample No	ELISA Titer*	SAT		
25	1	+	+	++	29	1	+	++	-	
26	1	+	++	++		6	+	++	+	
27	1	+	Nd	Nd		7	+	-	-	
	2	+	Nd	Nd	9	+	++	++		
28	1`	+	-	++	30	7	+	Nd	Nd	
	2	+	+	+		9	+	Nd	Nd	
	4	+	+	++						

SAT: Intensity of slide agglutination was represented by No. of (+);\* ELISA titer 1432 or greater/ each sample was considered positive for ORT antibodies according to manufacture procedure .Nd: Not done



**Figure 3: The comparative sensitivity of ELISA and SAT in detection of seroconversion against ORT in chicken broilers.**

Comparably, Lopes *et al.* [31] succeeded to detect antibodies using agglutination test in 65% of sera of infected birds within 2 weeks post infection which declined thereafter they explained that IgM antibodies which were efficient in agglutination specific antigens and appear in early stage of infection. In addition, most –reactors were serotype-specific, although cross-reactions can occur. They also revealed that ELISA can detect antibodies against ORT in the early period of infection up to 8 weeks of age. While titers were high between 1 to 4 weeks post infection and declined afterwards.

Also, Van Empel and Hafez [1] concluded that rapid agglutination test was more sensitive in early infection due to its better results with IgM than IgG. It was observed that the ORT -2 is more related to the detected antibodies in the examined sera more than ORT-1 and its sensitivity was close to ELISA test. But still ELISA the most sensitive one.

Being horizontally transmitted through direct and indirect contact via aerosols and / or drinking water [1] ORT could be endemic affecting every new restocking even in previously cleaned and disinfected houses. The obtained results in this study indicated early infection as young as 9 days of age broiler chickens and high percentage of detection 80% which support the concept of endemic status of ORT.

It could be concluded that ORT is one of the main components of respiratory disease complex among broiler chickens. In case of unavailability of ELISA, the SAT test using appropriate and commonly circulating serological antigen could be a useful. It has the advantages of being practical, low cost, and easy to use in the field. These features contribute to the speed of the results and the ability to take rapid action to field for monitoring of the disease especially in early stage of infection.

**Conflict of interest**

None of the authors have any conflict of interest to declare

**Acknowledgment**

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### الملخص العربي

#### مقارنة بين اختباري الاليزا و التلزن المصلي في الكشف عن الأجسام المضادة لبكتيريا الاورنيثو رينوتراكيا في دجاج التسمين بمحافظة الشرقية

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تشارك بكتريا الاورنيثو رينوتراكيا ((ORT في امراض الجهاز التنفسي المعقدة في الدواجن. في تجربة للتحقيق في الكشف عن الـ ORT في دجاج التسمين الذي يعاني من مشاكل في الجهاز التنفسي ، تم إخضاع مائتين وخمسين طائرًا (30 قطيعًا) للفحص الاكلينيكي والصفة التشريحية والاختبارات السيرولوجية. وتضمنت نتائج الفحص الاكلينيكي للطيور التي تم فحصها وجود إفرازات أنفية ، حشرجة ، تورم في العيون مصحوباً بالإسهال في بعض الحالات. وكانت التغيرات المرضية الاكثر تكراراً هي التهابات فبرينية في غشاء التامور وحول الكبد والتهاب الاكياس الهوائية وكذلك وجود افرازات متجبنة في القصبة الهوائية والشعب الهوائي. بجانب الافراز الرغوى الشبيه بالزبادي الرغوي والمصاحبة بشكل متكرر. تم استخدام اختبار الاليزا (ELISA) على عينات الأمصال (N = 250) لاكتشاف الأجسام المضادة ضد ORT. كانت نتائج ELISA ايجابية بين 80% من القطعان المختبرة ، كما تم اختيار عدد 49 عينة مصلية للخضوع لاختبارى ELISA واختبار التلزن المصلي على الشريحة (SAT). و أظهرت نتائجهم ايجابية 93.9% لـ ELISA و 89.8% و 69.38% ضد مستضدات ORT-I و ORT-II على التوالي. وعليه انه بالمقارنة يمكن في حال عدم توافر امكانيات اجراء اختبار ELISA ، يمكن أن يكون SAT بديلاً مفيداً في الكشف عن الاجسام المضادة لـ ORT.