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Use of Synthetic Antigens Improves Detection by Enzyme-Linked Immunosorbent Assay of Antibodies against Abortigenic *Chlamydia psittaci* in Ruminants†

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Synthetic peptide antigens were prepared for use in enzyme-linked immunosorbent assays (ELISAs) to detect serum antibodies against abortigenic strains of *Chlamydia psittaci* in livestock. Peptide antigens were identified with *C. psittaci* B577-immune sera by solid-phase scanning of overlapping octapeptides of variable domains (VDs) of the major outer membrane protein of *C. psittaci* serovar 1 (*omp1* type *C. psittaci* B577). Two VD 4 regions and one VD 2 region were strongly reactive with all *C. psittaci* B577 antisera. Peptides encompassing these regions were synthesized with biotin and a serine-glycine-serine-glycine spacer at the N terminus and were attached to streptavidin-coated microtiter plates. In direct ELISAs with these plates, the synthetic peptides reacted with *C. psittaci* B577 antisera, but not with sera from specific-pathogen-free animals. Serum specimens from 40 sheep and 40 cattle, obtained from herds with abortion problems, were screened for antibodies by these *C. psittaci* B577 peptide ELISAs and an ELISA with recombinant, genus-specific *Chlamydia* lipopolysaccharide (LPS) antigen. Results from these newly developed ELISAs were compared to those from the reference *C. psittaci* B577 elementary body (EB) ELISA and the *Chlamydia* complement fixation test (CFT). The *C. psittaci* B577 peptide ELISAs, the LPS ELISA, and the EB ELISA correctly identified the presence or absence of antibodies against chlamydiae in all sheep and bovine sera. The *Chlamydia* CFT, which is the most widely accepted serodiagnostic method for chlamydial infections in animals, correctly identified the presence or absence of antibodies against chlamydiae in only 78 and 4.9% of sheep and bovine sera, respectively. These results suggest that the *C. psittaci* B577-peptide and *Chlamydia* LPS ELISAs are superior for the serodiagnosis of ruminant infections with abortigenic chlamydiae, since they are more sensitive than the CFT, they are easy to standardize, and they use readily available synthetic antigens instead of organism-derived CFT antigen.

The eubacterial genus *Chlamydia* comprises obligate intracellular organisms which exhibit a two-stage life cycle that alternates between infective (elementary body [EB]) and vegetative (reticulate body) forms. The genus *Chlamydia* includes four species: *C. trachomatis*, *C. psittaci*, *C. pneumoniae*, and *C. pecorum* (5, 8, 13). These bacteria can cause many diseases in human and animal hosts, notably, ocular, urogenital, respiratory, and intestinal infections. *C. psittaci* is of particular interest in veterinary medicine because of its broad host range and the variety of diseases that it may cause in susceptible animals (15). Infection of ruminants with serovar 1 of *C. psittaci* (*omp1* type *C. psittaci* B577) is common and may result in enteritis, seminal vesiculitis, and abortion (1, 17, 18). The abortigenic potential of *C. psittaci* B577 applies not only to ruminants but also to humans with infections contracted from ruminants (1, 10).

Routine diagnosis of *Chlamydia* infection in animals is complicated by several factors. While isolation of the organism is preferable, it requires a facility experienced with tissue culture technique or chicken embryo culture. The serological diagnosis of chlamydial infection by the complement fixation test (CFT), EB enzyme-linked immunosorbent assay (ELISA), and the microimmunofluorescence test (MIF) is difficult to standardize and has traditionally been hindered by the cumbersome production of antigens in chlamydial culture. Additionally, the CFT and MIF are technically challenging and require skilled technicians to interpret the results. These problems restrict the use of CFT, EB ELISA, and MIF to a relatively small number of qualified laboratories. Therefore, simpler immunoassays with synthetic chlamydial antigens are needed.

Variable domains (VDs) 1, 2, and 4 of the major outer membrane protein (MOMP) contain the primary serovar-determining epitopes of chlamydiae (2, 4, 16). In this investigation, contiguous immunodominant peptide antigens of these VDs of the *C. psittaci* B577 MOMP were identified. Corresponding immunodominant peptides were then synthesized, and an ELISA method for the detection of antibodies against these antigens was established. In addition, a *Chlamydia* genus-specific ELISA was evaluated. The ELISA uses commercially produced microtiter plates coated with recombinant chlamydial lipopolysaccharide (LPS). Eighty sheep and cattle serum specimens obtained from herds with abortion problems were screened by these ELISAs. The peptide and LPS ELISA results were compared with those obtained by the *C. psittaci* B577 EB ELISA reference method (14) and by CFT, the most widely used diagnostic method.

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MATERIALS AND METHODS

Sera. The rabbit and murine *C. psittaci* B577-immune sera used in peptide scanning and establishing the *C. psittaci* B577 peptide ELISAs were kindly provided by J. Storz, Louisiana State University. Ovine sera used both in peptide scanning and in screening for antibodies were obtained from a flock in Austria after an episode of chlamydial abortion, as verified by *Chlamydia omp1* PCR, immunohistochemical staining, and isolation of chlamydiae (unpublished data). Bovine sera were obtained from herds in Alabama with abortion problems of unknown etiologies (kindly provided by Sara Rowe from sera submitted to the C. S. Roberts Veterinary Diagnostic Laboratory, Auburn, Ala.) and from the Large Animal Clinic at the Auburn University College of Veterinary Medicine, Auburn, Ala. Normal mouse sera and rabbit preinoculation sera were used as negative controls in establishing the peptide antigen ELISA. Immunoglobulin-rich sera from gnotobiotic animals challenged with a viral infection were used as negative controls in analyses of the bovine and ovine field serum specimens. For the sheep samples, immune serum from a lamb challenged with infectious bovine rhinotracheitis virus, kindly provided by D. R. Redman, Ohio State University, was used as a negative control. For the bovine samples, acute-phase immune sera from calves inoculated with bovine coronavirus or bovine diarrhea virus were used as negative controls. These sera were gifts from L. J. Saif, Ohio State University; G. E. Duhamel, University of Nebraska; and L. T. Nusz, National Animal Disease Center, Ames, Iowa.

Peptide scanning. Contiguous peptide epitopes of the *C. psittaci* B577 MOMP were identified by solid-phase peptide scanning with seven high-titer ovine, rabbit, and murine *C. psittaci* B577-immune serum specimens described above. Sequential octapeptides overlapping by seven residues and spanning VDs 1, 2, and 4, were synthesized by 9-fluorenylmethoxycarbonyl chemistry on polyethylene pins in a microtiter plate format (Multipin NCP; Chiron Mimotopes Peptide Systems, San Diego, Calif.). The degree of reactivity of these peptides with the *C. psittaci* B577-immune sera was analyzed by ELISA in a modified microplate format as described previously (7). The raw optical density (OD) values obtained for each peptide were corrected for background interference and unspecific conjugate binding and were transformed to percent above the baseline signal generated by the 10 peptides demonstrating the lowest reactivity.

Peptide ELISA. Immunodominant peptides suitable as ELISA antigens were synthesized with N-terminal biotin followed by a serine-glycine-serine-glycine spacer and the *Chlamydia*-specific peptide (Chiron). The amino acid sequences of the *Chlamydia*-derived peptide antigens used were as follows: *C. psittaci* B577 VD 2, QLPNVGITQGIV; *C. psittaci* B577 VD 4-1, LNLTTWNPTLLG; and *C. psittaci* B577 VD 4-2, ATALDTSNKFADFLQI. Fifty picomoles of peptides *C. psittaci* B577 VD 2, VD 4-1, or VD 4-2 were added per well to streptavidin-coated microtiter plates (11). The peptides were incubated for 1 h with 200 μl of Superblock reagent (Pierce, Rockford, Ill.) per well to block nonspecific binding, followed by five washes with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TTBS). Single 1:100 dilutions of sera in TTBS were incubated with the peptides for 1 h at room temperature on a rocker, and the wells were washed five times with TTBS. Anti-mouse, anti-rabbit, anti-sheep, or anti-bovine immunoglobulin–horseradish peroxidase conjugates were diluted in TTBS as follows: goat anti-mouse immunoglobulin (Boehringer Mannheim Corp., Indianapolis, Ind.), 1:2,000; goat anti-rabbit immunoglobulin G (IgG; Boehringer), 1:10,000; rabbit anti-sheep IgG (heavy and light chains; Pierce), 1:15,000; and rabbit anti-bovine IgG (Sigma Immunochemicals, St. Louis, Mo.), 1:15,000. Conjugates were incubated with the captured antibodies in microtiter wells for 1 h and were washed as described above, with a final wash with TBS only. Bound conjugate was detected by using tetramethylbenzidine substrate with a stopping reagent (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and by determining the spectrophotometric absorption at 450 nm. ODs were recorded after subtracting the mean background interference for each assay. Percent intra-assay variation was calculated as the average percent OD deviation for replicate specimens in an assay from the mean OD for these replicates. For duplicates of the assays, the ODs were normalized with the linear least-squares regression coefficients, and percent interassay variation was calculated as the average percent OD deviation from the mean of both assays.

***Chlamydia* LPS ELISA.** The *Chlamydia* LPS ELISA was performed as described above, except that microtiter plates coated with an artificial glycoconjugate antigen (Medac, Hamburg, Germany) were used instead of peptide-coated plates (3).

***C. psittaci* B577 EB ELISA.** Ovine serum specimens were evaluated by a modification of an ELISA with partially purified *C. psittaci* EBs used as antigen, as described previously (14). Rabbit anti-sheep IgG (heavy and light chains)–alkaline phosphatase (Kirkegaard & Perry Laboratories) was used at a dilution of 1:2,000 in TBS. After reaction with *p*-nitrophenylphosphate, bound conjugate was detected by measuring the absorption at 405 nm.

CFT. A micromethod of the Kolmer technique (14) was used to evaluate sheep sera at the Federal Institute for Control of Animal Diseases in Mödling, Austria. Guinea pig complement, *Chlamydia* CFT antigen, and hemolysin were obtained from Behringwerke AG, Marburg, Germany. Bovine sera were analyzed at the National Veterinary Services Laboratories Ornithosis Section (Ames, Iowa) with laboratory-produced reagents.

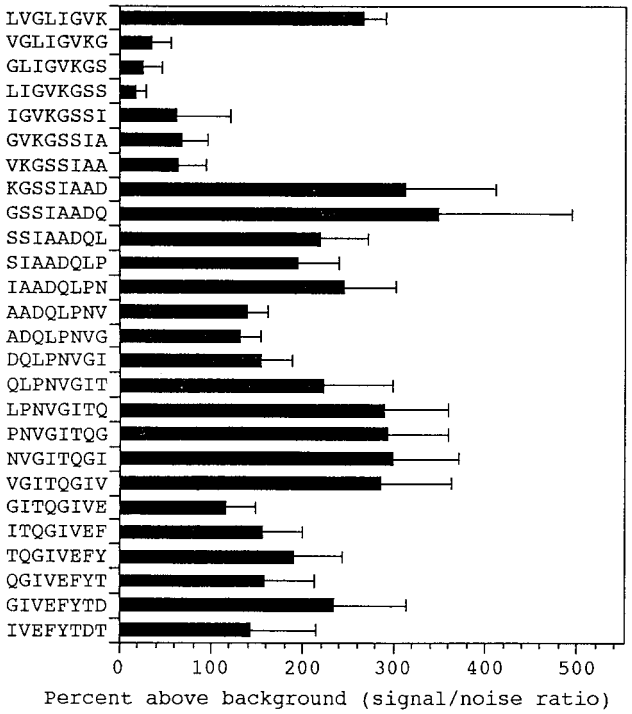

FIG. 1. Seroreactivity of *C. psittaci* B577 MOMP VD 2 octapeptides. Reactivities with seven high-titer ovine, rabbit, and murine *C. psittaci* B577-immune serum samples are plotted as percent above background (signal/noise ratio) for each peptide. The error bars represent the standard error of the mean for the population. Peptide scanning raw data were transformed to normalize absorption values between sera and to correct for nonspecific conjugate binding. For each serum sample, the mean of the 10 lowest OD values of all VD peptides was considered the background, and the percent above background (signal/noise ratio) of the transformed OD values was plotted. The C-terminal 12-mer peptide QLPNVGITQGIV was selected as an ELISA antigen because of the consistently high reactivity with all *C. psittaci* B577 antisera from all species tested.

RESULTS

***C. psittaci* B577 MOMP epitope scanning.** ELISA peptide antigens were defined by B-cell epitope scanning of VDs 1, 2, and 4 of the MOMP of *C. psittaci omp1* type *C. psittaci* B577. Highly reactive peptides were observed in all VDs. A VD 2 C-terminal peptide, QLPNVGITQGIV (Fig. 1), and two VD 4 peptides (Fig. 2), VD 4-1 (LNLTTWNPTLLG) and VD 4-2 (ATALDTSNKFADFLQI), reacted strongly with all *C. psittaci* B577 antisera and were selected as antigens. These peptides include several of the mapped octapeptides presented in Fig. 1 and 2, and they were chosen because they were reactive with all antisera, in contrast to some octapeptides which were highly reactive with some antisera but not reactive with other antisera. Such peptides with inconsistent reactivities, like peptide GSSIAADQ in VD 2 (Fig. 1), are characterized by a high standard error of the mean. Amino acid sequences of the VD 2 peptide are present only in ruminant and avian strains of *C. psittaci* (12), and thereby, the VD 2 peptide might serve as a reagent promising narrow specificity for the detection of infections with these organisms. Peptide VD 4-1 is highly conserved among *C. psittaci*, *C. pecorum*, and *C. pneumoniae*, and thus has potential for broad specificity. Conversely, amino acid sequences of peptide VD 4-2 are present only in ruminant and feline *C. psittaci* strains. While some sera reacted strongly with the VD 1 peptides, other sera did not react with the same peptides (data not shown). Because of this inconsistent re-

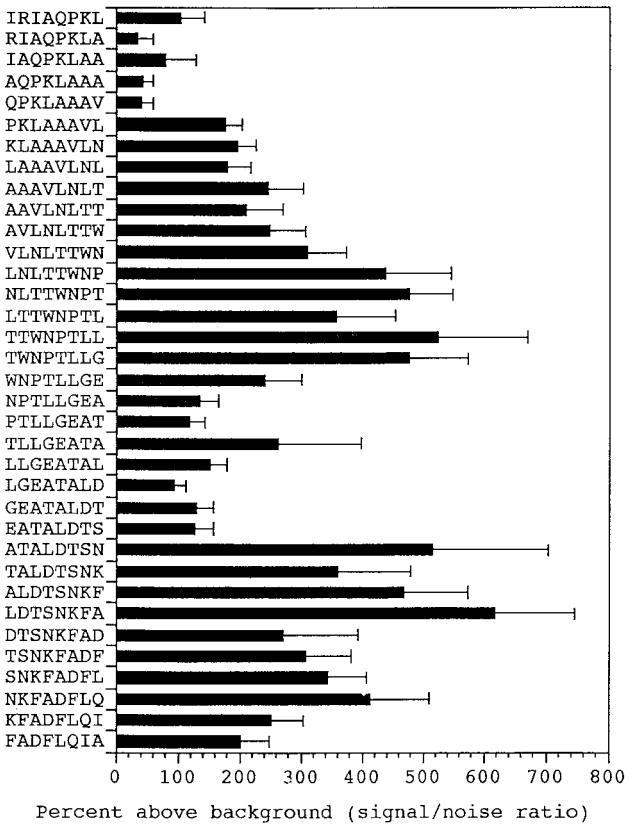

FIG. 2. Seroreactivity of *C. psittaci* B577 MOMP VD 4 octapeptides. Data are plotted in the same format described for Fig. 1. Two VD 4 peptides reacted strongly with all *C. psittaci* B577 antisera and were selected as ELISA antigens. The amino acid sequences of the VD 4 antigens are as follows: *C. psittaci* B577 VD 4-1, LNLTTWNPTLLG; *C. psittaci* B577 VD 4-2, ATALDTSNKFADFLQI.

activity, peptide epitopes of VD 1 were not selected as antigens.

***C. psittaci* B577 MOMP peptide ELISA.** An ELISA technique was established by using biotinylated peptide antigens attached to streptavidin-coated microtiter plates. Peptides *C. psittaci* B577 VD 2, VD 4-1, and VD 4-2 all reacted strongly with rabbit and murine *C. psittaci* B577-immune sera but not with negative control sera. Normal mouse serum and rabbit preinoculation sera (serum specimens 1 to 4 in Fig. 3) produced ODs of approximately 0.15 with all three peptide antigens, while the *C. psittaci* B577-immune sera except for serum specimen 8 with peptide VD 2 (serum specimens 5 to 10 in Fig. 3) produced higher ODs. The reactivity of rabbit immune sera against two serovars of *C. pecorum* (serum specimens 11 and 12 in Fig. 3) produced ODs minimally above the OD cutoff value of 0.2 for specific reactivity. Sequences of peptides VD 2 and VD 4-2 are unique for strains of *C. psittaci*, and thus, little or no cross-reactivity with *C. pecorum* had been anticipated. Peptide VD 4-1, however, is identical in *C. psittaci* B577 and all strains of *C. pecorum*, but *C. pecorum* antisera were essentially nonreactive. This observation parallels findings with *C. trachomatis*, in which the homologous peptide is conserved throughout the species but functions as a B-cell epitope only in certain serovars (2, 19).

Ovine and bovine serum specimens. Forty sheep serum specimens were first used to evaluate reactivities in the *C. psittaci* B577 peptide and *Chlamydia* LPS ELISAs versus the *C. psittaci* B577 EB ELISA reference method and the CFT. All sera reacted strongly with peptides *C. psittaci* B577 VD 2, VD 4-1, and VD 4-2 and with the *Chlamydia* LPS antigen. For all peptide and LPS ELISAs, the maximum averages of percent intra- and interassay variations were 7.6 and 8.5%, respectively. Negative control sera, when applied to the ELISAs, produced ODs of <0.1 and thereby were considered to lack reactivity.

All ovine serum specimens, but not the *Chlamydia*-negative ovine control serum, tested positive for specific antibodies in the *C. psittaci* B577 EB ELISA, although some of them reacted weakly (Fig. 4). Thus, positive and negative predictive values (6) of the *C. psittaci* B577 peptide and *Chlamydia* LPS ELISAs for the sheep specimens were 100%. The EB ELISA results were characterized by the serum specimens segregating into six groups ranging from low to high ODs, and thus, the values failed to correlate with the consistently high ODs that were obtained by peptide ELISAs and by the LPS ELISA in particular (Fig. 4). Thirty-one of 40 ovine serum specimens tested positive (≥1:10) by CFT, resulting in a positive predictive value of 100% and a negative predictive value of 10% for the CFT. Thus, the efficiency of the CFT in correctly classifying positive and negative antichlamydial seroreactivity in sheep was 78%. No correlation was observed between negative or low CFT titers and the OD values of the peptide, LPS, and EB ELISAs.

Following validation of the *C. psittaci* B577 peptide and *Chlamydia* LPS ELISAs, they were applied to tests with 40 bovine serum specimens obtained from herds with abortion problems of unknown etiology. The results of the ELISAs were compared to those of the CFT, which is widely used for the routine serodiagnosis of chlamydial infections in ruminants. While all bovine serum specimens were clearly reactive in the ELISAs, only one tested positive (≥1:10) by CFT (Fig. 5). Thus, the positive predictive value of the CFT for bovine sera was 100% and the negative predictive value was 2.5%. Overall, the results of the CFT correctly classified the presence or the absence of antibodies against chlamydiae in 4.9% of the bovine sera. Similar to the sheep sera, there was no correlation between the OD values of the three *C. psittaci* B577 peptide ELISAs and those of the *Chlamydia* LPS ELISA.

DISCUSSION

We have identified MOMP peptide epitopes of ruminant *C. psittaci* isolates which are suitable for use as reagents for the detection of antibodies against these chlamydiae. While reactivity of the *C. psittaci*-specific peptides *C. psittaci* B577 VD 2 and VD 4-2 with homologous antisera but not with heterologous *C. pecorum* antisera was expected, peptide *C. psittaci* B577 VD 4-1 unexpectedly was also nonreactive with the *C. pecorum* antisera. The hydrophobic peptide *C. psittaci* B577 VD 4-1 (LNLTTWNPTLLG) has an amino acid sequence that is highly conserved throughout the genus *Chlamydia*, and the motif LTTWNPTLLG is present in the MOMPs of *C. psittaci* B577 as well as all strains of *C. pecorum*. In *C. trachomatis*, the corresponding peptide is an immunodominant linear epitope in some serovars, while it is not antibody accessible in others (2, 4, 16, 19). Thus, differential folding of identical peptides might or might not render them linear epitopes. Also, amino acid changes frequently do not substantially alter the specificities of antibody-binding domains, presumably because of conserved conformation (9). Without extensive testing with antisera against a panel of serovars of all chlamydial species, the extent of cross-reactivity of the *C. psittaci* B577 peptide epitopes cannot be ascertained. Such studies should be per-

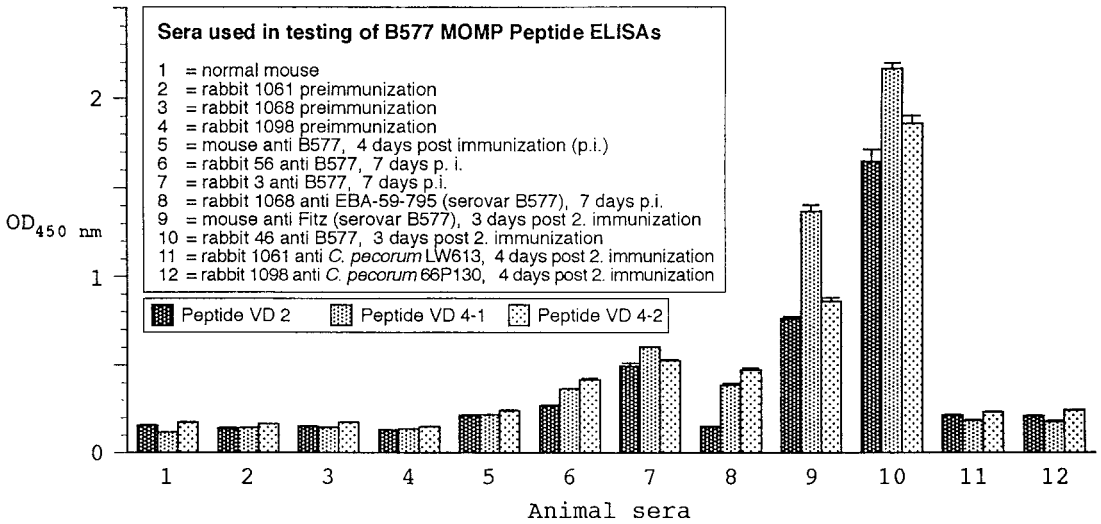

FIG. 3. Rabbit and mouse sera tested by ELISA with biotinylated *C. psittaci* B577 MOMP peptide antigens attached to streptavidin-coated microtiter plates. The means ± standard errors of the means of OD values of triplicate determinations for each peptide and animal are plotted. Samples 1 to 4, normal mouse and rabbit preinoculation sera; samples 5 to 10, *C. psittaci* B577-immune sera; samples 11 and 12, *C. pecorum*-immune sera. $OD_{450\ nm}$, OD at 450 nm.

formed to explore the full potential of peptide ELISAs for chlamydial seroepidemiology and to compare them to the MIF method for the serotyping of chlamydiae.

When the *C. psittaci* B577 peptide and *Chlamydia* LPS ELISAs were applied to specimens from ruminants in the field in this study, they demonstrated a very high prevalence of serum antibodies against chlamydiae and also high levels of these antibodies. We maximized the sensitivity of the detection system by using horseradish peroxidase conjugates in combination with the tetramethylbenzidine substrate and a stopping reagent. Saturation of the substrate reaction might have occurred in some assays, and therefore, even with resulting high OD values above 2.0, the OD values might not have reflected accurately the amount of antibody present. This may have been particularly true for the LPS ELISA with the sheep sera. Use of alkaline phosphatase conjugates and *p*-nitrophenylphosphate substrate might restore linearity at high absorption values.

The OD values with negative control ruminant sera were below 0.1 OD. Because of the ubiquity of antichlamydial antibodies in ruminants, selection of appropriate negative control sera was crucial. To best simulate samples from animals in the

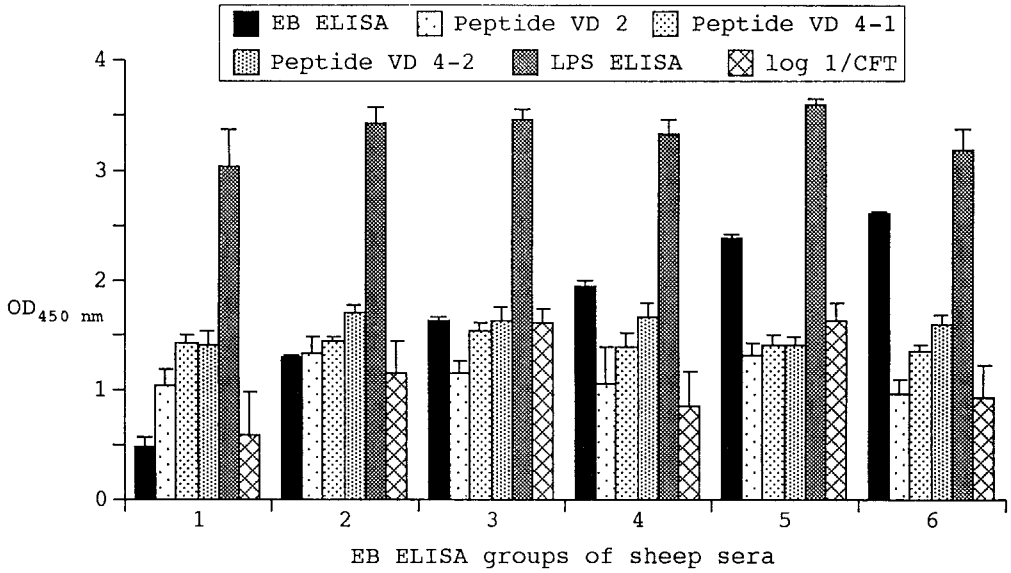

FIG. 4. Summary of serological data obtained from 40 ovine serum specimens from animals in the field. Ovine sera were placed in six groups according to their EB ELISA ODs. The mean ± standard error of the mean absorption at 450 nm ($OD_{450\ nm}$) corrected by subtracting background interference is shown for each group of EB ELISAs, *C. psittaci* B577 peptide ELISAs, and *Chlamydia* LPS ELISAs. CFT results are presented as the logarithm of the reciprocal CFT titer. Group 1 (n = 9), EB ELISA OD of ≤0.999; group 2 (n = 6), OD of 1.0 to 1.499; group 3 (n = 7), OD of 1.5 to 1.749; group 4 (n = 4), OD of 1.75 to 1.999; group 5 (n = 9), OD of 2.0 to 2.499; group 6 (n = 5), OD of ≥2.5.

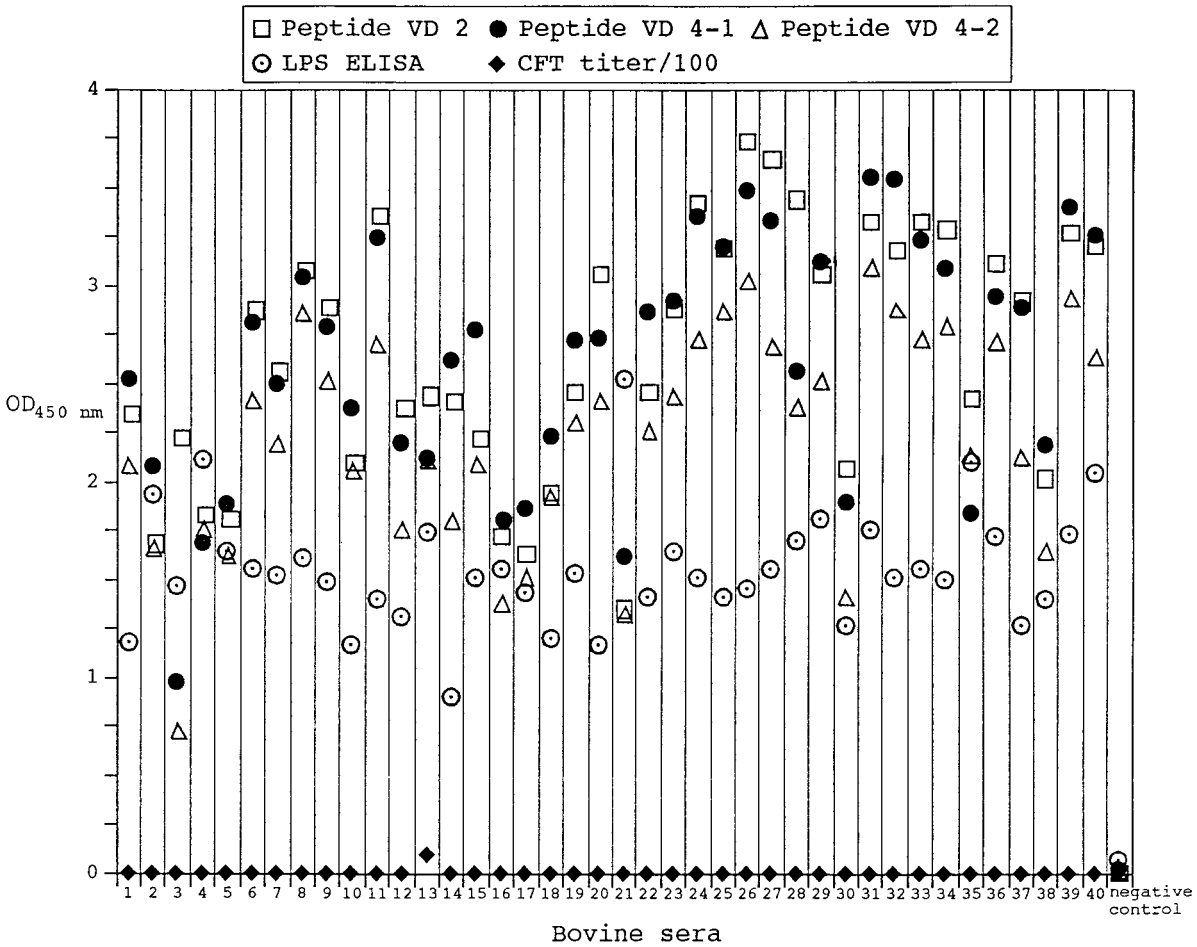

FIG. 5. Summary of serological data obtained from 40 bovine serum specimens from animals in the field. The OD at 450 nm ($OD_{450\ nm}$), as described in the legend to Fig. 4 for *C. psittaci* B577 peptide ELISAs, *Chlamydia* LPS ELISA, and CFT titer/100, is represented for each serum sample. A single bovine serum sample (sample 13) has a CFT titer of 1:10 above the negative baseline value. All ELISA methods yielded high OD values, while the negative control sample remained below 0.1 OD unit. The heights of the OD values of the LPS and all peptide ELISAs are not correlated.

field with high immunoglobulin content, we used sera from gnotobiotic animals after challenge with viral pathogens. The low absorption values of these sera confirmed that OD values of 0.15 and above represented specific reactivity with chlamydial antigens.

The comparison of data obtained from applying the peptide ELISA, LPS ELISA, EB ELISA, and CFT to ruminant serum specimens yielded several important observations. The ovine specimens segregated into six groups by the EB ELISA, reflective of low to high OD values (Fig. 4), but this segregation was not duplicated in the other test systems. Groups 1 and 2, with lower EB ELISA OD values, did have corresponding low CFT titers, but the correlation was not maintained for the other groups. The distribution of OD values of the peptide and LPS ELISAs was relatively consistent in all groups, but the values were not correlative between the assays. A similar lack of correlation was observed between all tests of bovine sera (Fig. 5). While this may imply an inability of the peptide and LPS ELISAs to correctly differentiate between higher and lower concentrations of serum antibodies, the more likely explanation is that (i) the EB ELISA and particularly the CFT are not sensitive enough to indicate the correct serum antibody concentration for some specimens, and (ii) the lack of correlation reflects the inherently variable antibody response against single epitopes versus those against the multiepitope, organismal antigens used in EB ELISA and CFT (3).

The obvious lack of sensitivity of the CFT, particularly for antibodies in the bovine sera, might be explained by (i) inconsistent quality of the antigen, (ii) detection of the complement-binding subset of immunoglobulin isotypes only, (iii) low level of production and/or short half-life of complement-fixing antibodies, and (iv) inefficient binding of the heterologous guinea pig complement by ruminant, particularly bovine, antibodies. In fact, Perez-Martinez et al. (14) have demonstrated that the addition of bovine complement greatly improved the sensitivity of CFT when applied to bovine sera.

While further testing of the peptide and LPS ELISAs at low and intermediate serum antibody concentrations is warranted, it is clear that use of the CFT as the standard test for detection of antibodies against chlamydiae in ruminants should be discontinued. It has poor efficiency, is technically challenging, is difficult to standardize, and cannot be automated. Results with the *C. psittaci* B577 peptide and *Chlamydia* LPS ELISAs suggest that they are superior replacements.

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